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Delayed Growth in Mycoheterotrophic Gametophytes of Seedless Vascular Plants

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Abstract.—Growth of the mycoheterotrophic gametophytes of *Sceptridium dissectum* and *Ophioglossum crotalophoroides* stalls at a few cells after germination on a nutrient medium lacking sugar. Resumption of growth when the cultures are supplied with glucose reveals that they are still alive. The stalled gametophytes of *S. dissectum* and *O. crotalophoroides* on media without sugar contain lipid droplets from the spores and a few starch grains that form during germination. The starch and lipid do not support the further growth of the stalled gametophytes because the size of the gametophytes does not change over time. These storage products slowly disappear from the stalled gametophytes. It appears that the storage materials are used to keep the stalled gametophytes alive. About 9% of the stalled gametophytes of *O. crotalophoroides* remain alive for ten months and about 5% of those of *S. dissectum* remain alive for 34 months. Delayed gametophyte growth is also exhibited by young gametophytes of *Botrypus virginianus*, *Lycopodium obscurum*, and *Psilotum nudum*. Delayed growth of mycoheterotrophic gametophytes under natural conditions would increase the time available for colonization by appropriate mycorrhizal fungi.

Key Words.—Ophioglossales, Lycopodiales, Psilotales, mycorrhiza, sugar-free medium

Spores from plants with mycoheterotrophic gametophytes germinate in the dark (Whittier, 1973, 1998; Whittier and Braggins, 1994) whether on a nutrient medium in culture or embedded in soil (Whittier, 1984). The spores require the absence of light or more specifically the absence of red light (Whittier, 2006, 2008) before germination occurs. The spores of *Ophioglossum crotalophoroides* Walt. and *Sceptridium dissectum* (Spreng.) Lyon are trilete and contain significant amounts of lipid. In *S. dissectum*, lipid makes up 32% of the weight of the dry spores (Melan, 1985). Starch is not present in the dry spores. However, once the spores are wet and have swollen starch appears before the triradiate ridge has cracked open. *Sceptridium* spores germinate on nutrient media with or without sugar. However, on a medium without sugar, gametophytes form only a few cells and bulge only slightly from the spore coats. The gametophytes reach maturity only on a nutrient medium with sugar (Whittier, 1972, 2003). The small gametophytes that form on a nutrient medium without soluble carbohydrates do not appear to die when their growth stalls. These gametophytes contain lipid but it does not seem to be used for continued gametophyte growth on a medium lacking sugar (Whittier, 1984).

The mycorrhizal symbionts of *Botrychium* were characterized by Winther and Friedman (2007) at three stages in the life cycle of *Botrychium*. Two stages include the subterranean gametophytes and the young subterranean sporophytes that are completely dependent on the symbionts for fixed carbon. The

last stage includes the symbionts of the photosynthetic sporophytes that are assumed to have a mutualistic relationship with the host sporophytes. Because the gametophytes are totally dependent on the mycorrhizal fungi, they must be colonized early in their development. There is evidence that small few-celled gametophytes of *Ophioglossum* are colonized by mycorrhizal fungi before subsequent gametophyte growth occurs. This raises the question of how long these non-growing, few-celled mycoheterotrophic gametophytes (stalled gametophytes) can remain alive. The objective of this study is to examine whether these stalled mycoheterotrophic gametophytes can remain alive for a long time and whether they can resume growth if soluble carbohydrates are made available. Finally it would be of interest to determine whether delayed gametophyte growth occurs in any of the other groups of seedless vascular plants with mycoheterotrophic gametophytes.

Materials and Methods

Species investigated for the study were: *Ophioglossum crotalophoroides* Walt., *Sceptridium dissectum* (Spreng.) Lyon, *Botrypus virginianus* (L.) Michx., *Lycopodium obscurum* L., and *Psilotum nudum* (L.) Pal. Beauv. The nomenclature for *Botrychium* used in this report follows Barker and Hauk (2003), that is *Sceptridium dissectum* (=*Botrychium dissectum*) and *Botrypus virginianus* (=*Botrychium virginianum*). Spores were collected in Tennessee except for those of *O. crotalophoroides* from Alabama and those of *P. nudum* from the greenhouse plants at Vanderbilt University, Nashville. Gametophytes of these five species require mycorrhizal symbionts to provide the organic carbon for their growth in nature. These symbionts have been identified using DNA data as *Glomus* spp. (Glomeromycota) for *Botrychium* and *Lycopodium* (Winther and Friedman, 2007, 2008).

The nutrient medium contained 100 mg $MgSO_4 \cdot 7H_2O$, 40 mg $CaCl_2$, 100 mg K_2HPO_4, and 100 mg NH_4Cl per liter. In addition, 0.5 ml of a minor element solution (Whittier and Steeves, 1960) and 4 ml of a FeEDTA solution (Sheat *et al.*, 1959) were added. The control medium contained 0.2% glucose and the experimental medium lacked glucose. Both media were adjusted to pH 6.0 before autoclaving and they were solidified with 1.1% agar.

The spores were surface sterilized with 1.1% sodium hypochlorite (Whittier, 1964), suspended in sterile water and sown on 12 ml of nutrient medium in culture tubes (20 $\times$ 125 mm) with screw caps that were tightened after inoculation. The cultures were maintained at $22 \pm 1^{\circ}$ in the dark. After the spores were sown, the culture tubes were covered with aluminum foil before being placed in the dark. At irregular intervals after germination had reached its maximum level (unpub. data) additions of glucose were made to the experimental cultures. The additions were made at 4, 6, and 8 months for *O. crotalophoroides* and at 7, 14, and 32 months for *S. dissectum*. In all cases the cultures were examined two months later for gametophyte growth. The additions were made to the foil covered tubes under dim green light (Pratt, 1973) to reduce any possible effects that short exposures of dim light would

have on the stalled gametophytes. Sterile water (0.7 ml) or a sterile 0.5% glucose solution (0.7 ml) or nothing was added to the surface of glucose-absent cultures. The 0.5% glucose solution rather than a 0.25% glucose solution was used for sugar addition to the glucose-absent culture to reduce any dilution problems. The stalled gametophytes were exposed to the dim green light for about 30 s while the addition was being made. The caps were replaced and the tubes were returned to the dark for two months. No additions were made to the tubes with the control medium already containing 0.2% glucose.

After two months, the contents of the tubes with or without the additions were removed to ascertain the percentage of germination, gametophyte cell numbers, gametophyte lengths, and storage contents of the gametophyte cells. Gametophyte length was measured from the base of the spore coat, or if absent, from the base of the gametophyte to the apex of the gametophyte. Stained gametophytes were treated with acetocarmine for nuclei, I_2KI for starch, or Sudan IV for lipid (Jensen, 1962). The sample sizes were 500 for the germination percentages, 200 for the gametophyte cell numbers, and 30 for the gametophyte length. The standard deviations were included with the averages for the cell numbers and gametophyte lengths. Gametophytes were considered to be growing if they had not turned brown and were larger than the stalled gametophytes.

Spores of *Botrypus virginianus, L. obscurum*, and *P. nudum* were germinated in the dark on a nutrient medium lacking glucose. Spores of *B. virginianus* and *L. obscurum* were examined at 14 months and those of *P. nudum* were examined at 9 months for gametophyte growth.

RESULTS

The germination process was the same for *O. crotalophoroides* and *S. dissectum* although the timing was different. Earliest germination occurred within two weeks for the spores of *O. crotalophoroides* and about six weeks for those of *S. dissectum* (unpub. data).

Ophioglossum crotalophoroides.—Germination (98%) was achieved by six months in this experiment whether the spores were sown on media with or without glucose. Because almost all the spores germinated initially subsequent germination was insubstantial. The spores of *O. crotalophoroides* germinated on the nutrient media with or without glucose (Table 1). Growth of gametophytes on the medium without glucose stalled (Fig. 1A) shortly after the spores germinated. The average cell number for the stalled gametophytes was 5.3 ± 0.6 with 7% having four cells, with 70% having five cells, 17% having six cells, and 6% having seven cells. The average cell number for the stalled gametophytes was the same throughout the experiment. Gametophyte growth on the medium with glucose for the whole experiment did not stall (Fig. 1B) but increased in size and cell number (Table 1).

Stalled gametophytes in cultures initially without sugar that had glucose added for the last two months before being examined produced more than 30 cells and grew to approximately double the size of the stalled gametophytes

TABLE 1. Delayed gametophyte growth in *Ophioglossum crotalophoroides*.

	Nutrient medium without glucose[1]								
	Nothing added last 2 mo			Water added last 2 mo			Glucose added last 2 mo		
Time from sowing spores	6 mo	8 mo	10 mo	6 mo	8 mo	10 mo	6 mo	8 mo	10 mo
Spore germination	98.7			98.2			98.5		
Gametophyte length (µm)	77.9±5.9	77.2±5.8	81.2±6.4	78.8± 7.4	81.8± 6.8	82.7± 5.6	186.9±26.3	182.0±25.4	176.3±30.9
Growing gametophytes (%)	0.0	0.0	0.0	0.0	0.0	0.0	99.5	45.6	9.3
Stalled gametophytes (%)	100.0	100.0	100.0	100.0	100.0	100.0	0.0	51.2	86.3
with starch (%)	15.6	0.2	0.0	12.8	0.1	0.0	-[2]	-[2]	-[2]
with lipid (%)	71.4	15.7	1.5	75.8	17.2	1.1	-[2]	-[2]	-[2]

[1] No stalled gametophytes occurred when spores were sown on a medium with 0.2% glucose. All the gametophytes grew and they attained an average size of 942.7 ± 307 µm at 10 months. [2] No data taken. The small number of gametophytes that could not be classified as stalled or growing was omitted from the results. mo = months.

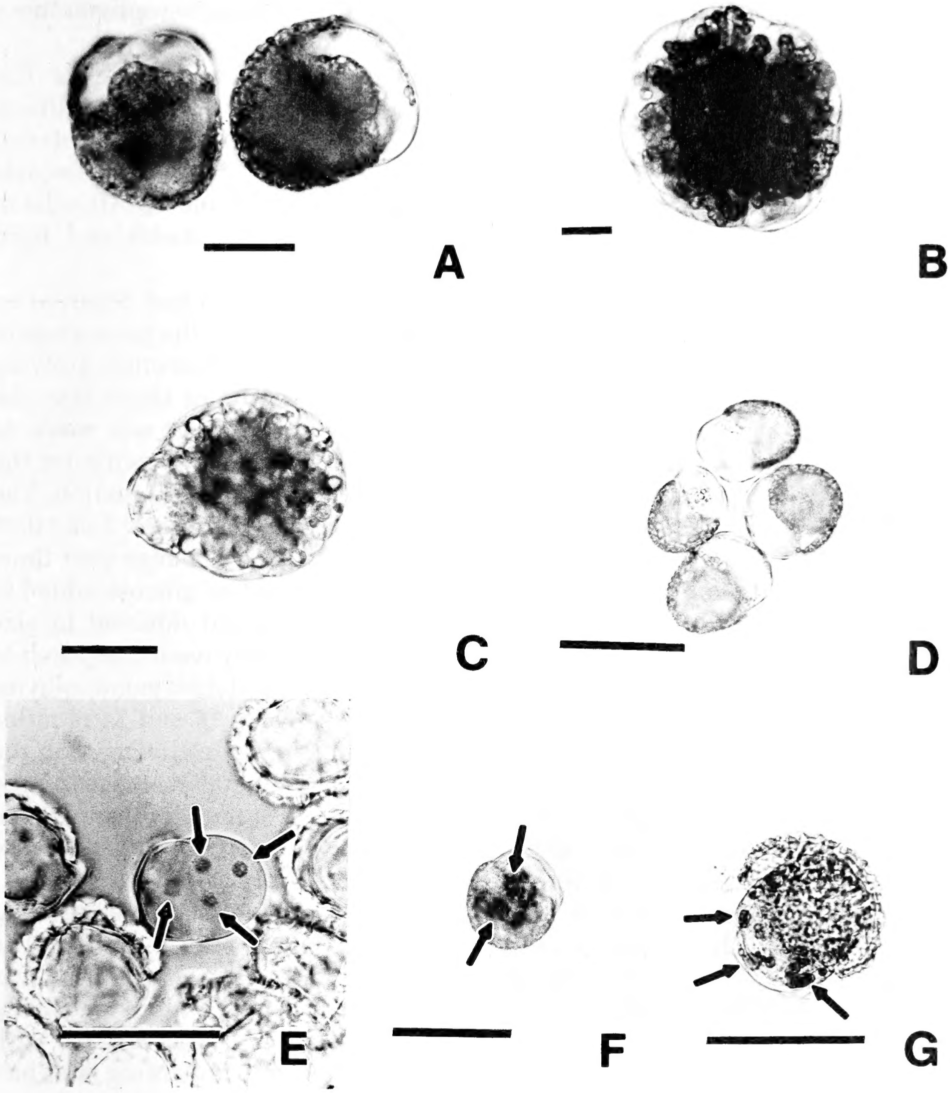

FIG. 1. Young gametophytes of *Ophioglossum* and *Sceptridium*. A–B, *O. crotalophoroides*. A) Stalled gametophytes. B) Growing gametophyte. C–G, *S. dissectum*. C) Growing gametophyte. D) Stalled gametophytes. E) Stalled gametophyte with four stained nuclei (arrows). F) Starch grains (arrows) in stalled gametophyte. G) Lipid droplets (arrows) in stalled gametophyte. Scale bars = 50 µm.

not supplied with glucose. Likewise these gametophytes on the medium with glucose for ten months were over ten times longer than the stalled gametophytes on a medium without glucose (Table 1).

On either medium, starch initially appeared as small grains next to the nuclei at germination. This starch essentially disappeared by eight months in

the stalled gametophytes and never disappeared in the gametophytes on a glucose medium.

Lipid droplets are present in the spores and in all young gametophytes. The lipid droplets were retained in stalled gametophytes on the medium without glucose longer than the starch (Table 1). However, with time, fewer stalled gametophytes contained lipid droplets. At six months 76% had lipid droplets whereas at ten months less than 2% had lipid droplets (Table 1). All cells of growing gametophytes on media with glucose contained starch and lipid droplets.

Sceptridium dissectum.—Germination of slightly over 80% had occurred by nine months on all media (Table 2).There was little change in the percentage of germination for the remaining time. Gametophytes of *S. dissectum* growing (Fig. 1C) on the medium with glucose were about seven times larger than the stalled gametophytes on a medium without glucose. No effort was made to determine their cell number. Gametophytes of *S. dissectum* growing on the medium without glucose stopped growing shortly after spore germination. The average cell number of these stalled gametophytes (Fig. 1D) was 3.98 ± 0.25 with 95% of them having four cells (Fig. 1E) and it did not change over time. Similar to *O. crotalophoroides*, stalled gametophytes that had glucose added to the culture for the last two months before being examined doubled in size compared to those on the medium without glucose and they were composed of 40 or more cells. The starch (Fig. 1F) that appeared in the stalled gametophytes at the time of germination disappeared sometime between 16 and 34 months. Lipid droplets (Fig. 1G) remained in the stalled gametophytes longer than the starch (Table 2). As the stalled gametophytes aged the percentage of the gametophytes containing lipids decreased from 93% at nine months to 7% at 34 months (Table 2). All gametophytes growing on a medium with glucose contained starch and lipid during the 34 months of the experiment.

Other species.—Stalled gametophytes with storage materials were observed for three additional species: *B. virginianus*, *L. obscurum*, and *P. nudum*. The average number of cells for the stalled gametophytes of *B. virginianus* was 3.8 ± 0.4 with 85% having four cells and the remaining gametophytes having two or three cells. For *L. obscurum* the average cell number was 3.1 ± 0.5 with 81% of the stalled gametophytes having three cells and the remaining ones had two, four, or five cells. Those of *P. nudum* had an average cell number of 1.9 ± 0.2 with 94% having two cells and 6% having one cell.

Discussion

Gametophyte growth of *O. crotalophoroides* and *S. dissectum* stalls after a few cells on a medium lacking soluble carbohydrates. Gametophytes of *B. virginianus*, *L. obscurum*, and *P. nudum* also stop growing under the same conditions. Stalling of gametophyte growth appears to be a general phenomenon for mycoheterotrophic gametophytes of seedless vascular plants. These small, non-growing gametophytes are not dead but living in a stalled state with growth resuming when soluble carbohydrates are added.

TABLE 2. Delayed gametophyte growth in *Sceptridium dissectum*.

	Nutrient medium without glucose[1]								
	Nothing added last 2 mo			Water added last 2 mo			Glucose added last 2 mo		
Time from sowing spores	9 mo	16 mo	34 mo	9 mo	16 mo	34 mo	9 mo	16 mo	34 mo
Spore germination	79.3	79.1	79.5	83.9	78.8	81.7	84.1	78.1	79.8
Gametophyte length (µm)	42.9±3.7	42.7±3.8	43.3±2.7	43.1±3.8	42.1±3.5	41.3±2.4	96.7±16.1	94.9±18.7	88.2±11.4
Growing gametophytes (%)	0.0	0.0	0.0	0.0	0.0	0.0	98.1	92.2	15.5
Stalled gametophytes (%)	100.0	100.0	100.0	100.0	100.0	100.0	0.0	5.1	82.2
with starch (%)	43.5	12.8	0.0	48.1	15.1	0.0	-[2]	-[2]	-[2]
with lipid (%)	93.1	58.9	11.4	92.6	70.3	7.0	-[2]	-[2]	-[2]

[1] No stalled gametophytes occurred when spores were sown on a medium with 0.2% glucose. All the gametophytes grew and they attained an average size of 1104 ± 408 µm at 34 months. [2] No data taken. The small number of gametophytes that could not be classified as stalled or growing was omitted from the results. mo = months.

In earlier studies on *S. dissectum* (Whittier, 1984) it was shown that the absence of soluble carbohydrates stopped the growth of young and mature gametophytes. Both young and mature gametophytes were unable to use external insoluble carbohydrates (starch and cellulose) for growth (Whittier, 1984). The use of sterile soil instead of a nutrient medium without sugar had the same effect on the young gametophytes. This would be expected because it has been concluded by Smith (1966) and Stevenson (1994) that there are basically no soluble carbohydrates available for plants in soil. Gametophyte growth stalls in the absence of a soluble carbohydrate in culture or without nutrients supplied by a mycorrhizal fungus in nature.

The young gametophytes seem unable to use their storage materials for extended gametophyte growth. The stalled gametophytes of *O. crotalophoroides* and *S. dissectum* contain ample storage materials in the form of starch and lipid droplets. These storage materials would be expected to support the gametophyte growth but they do not, as revealed by the unchanged size of the stalled gametophytes after ten months for *O. crotalophoroides* and 34 months with *S. dissectum*. However, the storage materials appear to be used to keep the cells of the stalled gametophytes alive.

The delayed growth of these fern gametophytes is similar to a condition found in the early development of orchids (Arditti, 1979; Smith and Read, 1997). Orchid embryos under asymbiotic conditions, like these fern gametophytes, need sugar for their continued early growth. For orchids, continued growth beyond the early protocorm stage needs soluble carbohydrates. Orchid embryos in their early protocorm stage only utilize their lipid storage materials very slowly (Arditti, 1979). They cannot use externally supplied large molecules like starch and cellulose. These seedlings, which cannot use their storage materials for growth, come to a 'resting stage' and remain alive by slowly utilizing their storage products. In the simplest case this 'resting stage' continues until soluble sugars are available for growth. The sugar necessary for growth is provided by mycorrhizal fungi in soil and by an appropriate nutrient medium in culture. The 'resting stage' (Arditti, 1979) or 'waiting time' as noted by Rasmussen (1995) and reported by Smith and Read (1997) of orchid embryos is similar to the 'stalled' state of mycoheterotrophic fern gametophytes.

Although the storage materials in the stalled gametophytes (starch and lipids) do not support further gametophyte growth, they do disappear from these gametophytes over time (Tables 1, 2). Starch is lost faster than the lipid (Tables 1, 2). Many gametophytes retain the storage products, especially the lipid droplets, for considerable lengths of time. A few of the stalled gametophytes of *O. crotalophoroides* had lipid at ten months; the comparable time for *S. dissectum* was 34 months (Tables 1, 2). Stalled gametophytes without storage products increased in number over time and they usually turned brown. These gametophytes were considered to be dead because they did not resume growth if supplied with sugar.

Fewer stalled gametophytes with storage products were present as time in culture increased and fewer gametophytes could be stimulated to grow. By the

time all the stalled gametophytes had run out of storage products, none would have the potential to resume growth whether it involved the addition of soluble carbohydrates to the cultures or colonization by a mycorrhizal fungus under natural conditions. Species with stalled gametophytes that used their storage products slowly would have an advantage over other species with gametophytes that used their storage products more rapidly. A longer time period increases the probability of colonization by mycorrhizae and subsequent growth.

Campbell (1907) reported that young gametophytes of *Ophioglossum* did not continue to grow until they were colonized by a mycorrhizal fungus. Bruchmann (1910) observed a similar situation with young *Lycopodium* gametophytes. This is consistent with what has been found for *Botrychium* and *Ophioglossum* gametophytes in culture. The stalled gametophytes can remain alive by using their storage materials for extended periods of time, but additional growth only occurs after soluble carbohydrates are provided.

Photoinhibition of spore germination insures that spores of species with mycoheterotrophic gametophytes will only germinate underground (Whittier, 2006, 2008). The subterranean germination of these spores would improve the possibility of sufficient moisture for gametophyte growth and development. These spores in the soil would be a temporary component of a spore bank (Sharpe and Mehltreter, 2010) because they germinate in the dark. Once germination and gametophyte growth occurs, they would be considered as belonging to a gametophyte bank (Farrar *et al.*, 2008) or to the belowground structure bank recognized for *Botrychium* (Johnson-Groh *et al.*, 2002).

The subterranean germination insures that the gametophytes develop underground and it enhances the possibility that young gametophytes will be in the proximity of mycorrhizal fungi. Delayed gametophyte growth has the potential to give the gametophytes more time for colonization. The use of the storage materials for increased cell growth likely has a shorter time frame than keeping the small but stalled gametophytes alive for a long time. The presence of small, but living gametophytes in the soil would improve the possibility for colonization to occur and mature gametophytes to develop.

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A Systematic Study of the Fern Genus *Mesopteris* Ching (Thelypteridaceae)

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Abstract.—The systematic position of the fern genus *Mesopteris* is described and discussed based on its sole species, *M. tonkinensis*. Evidence for its generic separation is based on its gross morphological characteristics, leaf epidermis, gametophyte development, spore morphology, and chromosome numbers. A morphological comparison with other related genera of *Amphineuron*, *Cyclosorus*, *Dryopteris*, *Lastrea*, and *Thelypteris* is provided.

Key Words.—morphological character, systematic position, *Mesopteris*, Thelypteridaceae

Dryopteris tonkinensis C. Chr. (Dryopteridaceae) was published in 1934, and was later treated as a member of various different genera, including as *Thelypteris tonkinensis* (C. Chr.) Ching (Thelypteridaceae) in 1936, *Lastrea tonkinensis* (C. Chr.) Copeland (Thelypteridaceae) in 1947, and *Amphineuron tonkinense* (C. Chr.) Holttum (Thelypteridaceae) in 1977. Ching (1978) established a new genus *Mesopteris* (Thelypteridaceae) based on the monotypic *M. tonkinensis* (C. Chr.) Ching. The molecular, phylogenetic analysis of He and Zhang (2012) supported *M. tonkinensis* as a part of the *Cyclosorus* clade. This species is distributed in a few locations in Guangxi, China, and northern Vietnam (Fig. 1). Few studies have been done on the morphology and cytology of the species because of its inaccessibility (Huang and Zhou, 1994). The aims of this paper are to provide morphological and cytological data, and to discuss the systematic position of *M. tonkinensis*.

Materials and Methods

Living plants of *Mesopteris tonkinensis* were collected from Longzhou, Daxin, and Napo County, Guangxi Province, China, and cultivated in the greenhouse of Guangxi Normal University. Voucher specimens (Table 1) were

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FIG. 1. The distribution of *Mesopteris tonkinensis*.

deposited in the herbarium of Guangxi Institute of Botany, Chinese Academy of Sciences (IBG).

Leaf epidermis.—Leaves were washed in water before being macerated in 5% NaOH solution for 10–24 hours. Pieces of leaf epidermises were washed in distilled water. To survey the constancy of epidermal structure, at least five slides were made from different parts of each leaf.

Spore morphology.—Mature spores were gathered from the specimens listed in Table 1. Materials for observation under SEM were directly affixed on stubs by double-sided adhesive tape, without further treatment. All preparations were coated with gold (using IB-5 sputter-coater) and photographed with AMARY-1000B SEM. At least 30 spores were counted in each individual under SEM.

Gametophyte development.—Spores were sown in improved Knop's agar medium (Liu *et al.*, 1991) and distilled water (for easier access of filaments). The pH of the Knop's medium was 6.3–6.5. Before sowing, spores were sterilized with 5% NaClO solution for 10–15 mins, and then rinsed with sterilized water three times. Cultures were kept at 25°C under a 12-hour photoperiod, a photon flux density of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (provided by cool white fluorescent tubes) and 90% relative humidity.

TABLE 1. Source information for plant materials.

Collection no.	Location	Individuals	Elevation	Plant materials	Date collected
Wang RX-0301	Longzhou, Guangxi	4	210 m	Chromosome and leaf epidermis	May 2011
Wang RX-0801	Longzhou, Guangxi	2	230 m	Chromosome and gametophyte	October 2012
Wang RX-1001	Daxin, Guangxi	3	850 m	Leaf epidermis	May 2011
Wang RX-0802	Napo, Guangxi	5	1005 m	Spore and gametophyte	September 2012

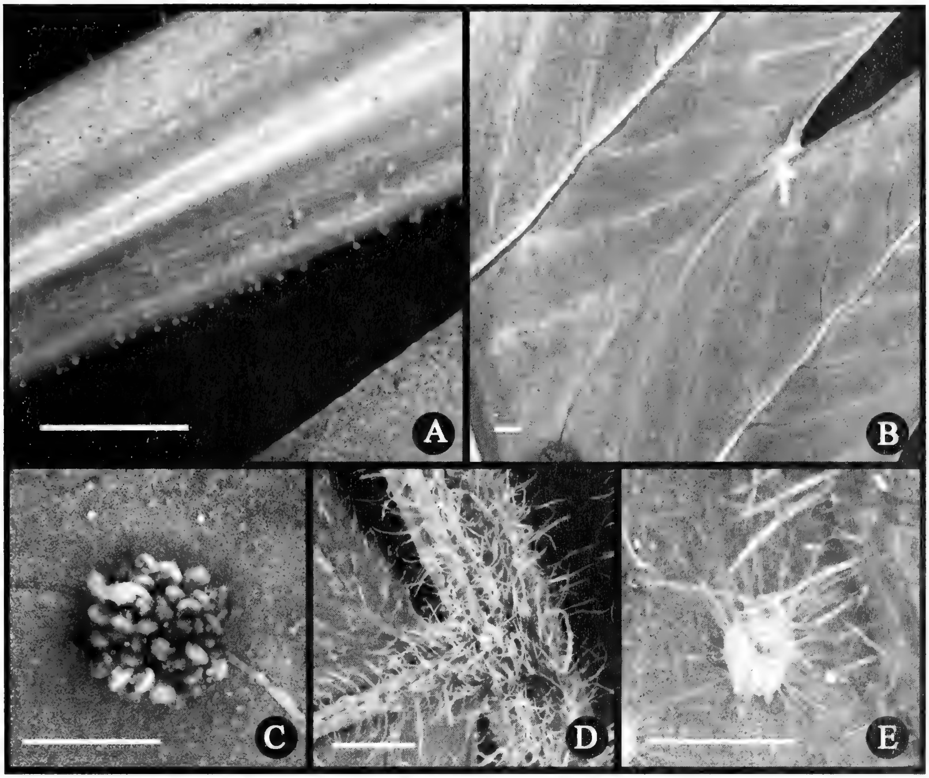

FIG. 2. Gross morphology of *Mesopteris tonkinensis* (A–C) and *Cyclosorus parasiticus* (D–E). A, D. Rachis. B. Venation (arrow: arch-shaped projections). C, E. Sorus. Scale bar =1 mm (A–E).

Cytological observation.—Living plants (Table 1) for cytological examination were collected in the fields of Longzhou, and cultivated in the greenhouse. Root tips were pretreated in 0.002 mol/L 8-hydroxyquinoline solution for 5–6 hours before being fixed in Carnoy's solution for 18–24 hours; then they were hydrolyzed in 1 mol/L HCl at 60 °C for 13–15 min. After washing 3–4 times to eliminate residual hydrochloric acid, materials were stained in 2% aceto-orcein and squashed following the method of Wang *et al.* (2011). More than 50 mitotic cells from 15 root tips representing six individuals were counted for the chromosome numbers. To obtain meiotic chromosome counts, suitable pinnae (on which the sori were slightly yellow) were fixed in Carnoy's solution for 18–24 hours; then stained in 2% aceto-orcein and squashed following the method of Wang *et al.* (2011). More than 30 sporangial meiotic cells at late diplotene were counted. To determine the reproductive type, the number of spores per sporangium was counted from at least five unopened sporangia from each of five individuals. The materials were examined and photographed by using a Nikon DXM 1200C camera.

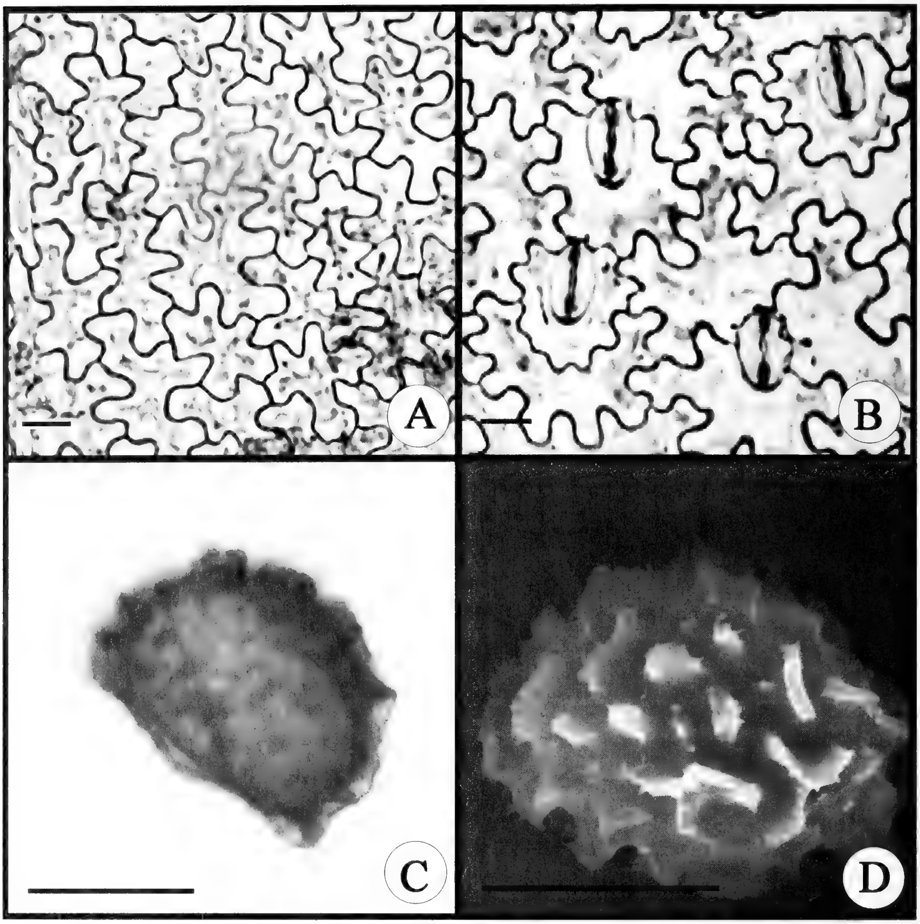

FIG. 3. Leaf epidermises and spore morphology of *Mesopteris tonkinensis*. A. Adaxial epidermis. B. Abaxial epidermis. C–D. Spore observation (C: light microscope; D: under SEM). Scale bar=20 μm (A–D).

RESULTS AND DISCUSSION

A molecular phylogenetic study using sequences of three cpDNA loci revealed the polyphyly of Thelypteridaceae. Species from 14 genera comprised a monophyletic *Cyclosorus* clade. Three accessions of *Mesopteris tonkinensis* were sampled, and all three formed a well-supported clade (BP >95%) within the *Cyclosorus* clade (He and Zhang, 2012). Furthermore, few cladistic analyses of morphological and cytotaxonomic data have been reported, which makes the phylogenetic relationships within the family difficult to resolve. Based on the reasons above, we make morphological comparisons to the related genera of *Amphineuron*, *Cyclosorus*, *Dryopteris*, *Lastrea*, and *Thelypteris*, and surveyed the main characters of *Mesopteris tonkinensis*, and then discuss the systematic position of *Mesopteris*.

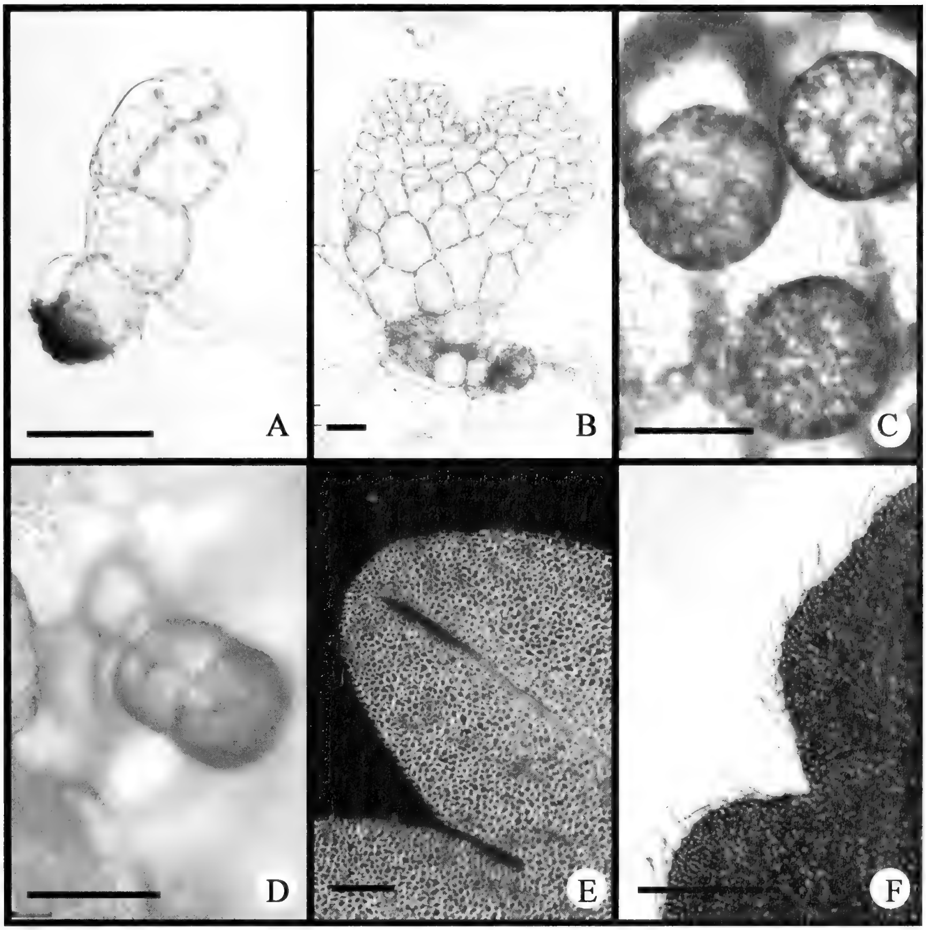

FIG. 4. Gametophyte development and young sporophyte of *Mesopteris tonkinensis* (A–E), young sporophyte of *Cyclosorus parasiticus* (F). A. 6-celled filament, 12 days after germination. B. Young gametophyte, 21 days after germination. C. Antheridia, 35 days after germination. D. Archegonium, 42 days after germination. E–F. Young sporophyte. Scale bar=40 μm (A–D); 1 mm (E–F).

Main diagnostic characters.—Seventeen specimens from both the field and the herbarium were examined. *Mesopteris tonkinensis* can be identified as follows: petiole and frond surface covered with golden brown glandular hairs (Fig. 2A), arch-shaped projections in the connection of two neighboring veinlets (Fig. 2B), and sori round, glabrous, and exindusiate (Fig. 2C). Specimens collected from different seasons were checked to ensure the stability of indusium characters. Lack of indusia makes *Mesopteris* distinct from other genera. For example, the petiole and frond surface (Fig. 2D) and the indusium (Fig. 2E) of *Cyclosorus parasiticus* are covered with numerous hispid and long flexible hairs.

Leaf epidermis observation.—The leaf epidermal cells of *Mesopteris tonkinensis* were usually irregular in shape. The patterns of anticlinal walls

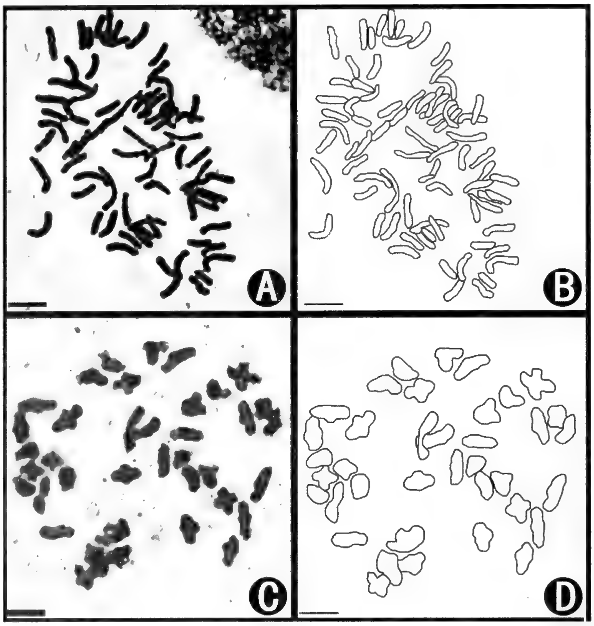

FIG. 5. Photographs of chromosomes and white-black lined drawing of *Mesopteris tonkinensis*. A–B. Metaphase chromosome, $2n=76$. C–D. Meiosis chromosome, $n=38$. Scale bar=10 μm (A–D).

were sinuate (Fig. 3A). The stomatal apparatus was restricted to abaxial epidermises and generally could be described as polocytic, coaxillocytic, or aisocytic types (Fig. 3B).

Spore morphology.—The spores of *Mesopteris tonkinensis* were brown, monolete, bilaterally symmetric, ellipsoidal in polar view, and semicircular shaped in equatorial view (Fig. 3C). Spores had prominent wing-like cristae with few echinae on the cristae (Fig. 3D). Related genera exhibit different spore surface ornamentations, such as lophate in *Cyclosorus* (Dai *et al.*, 2002), tuberculate in *Amphineuron*, verrucate in *Dryopteris*, granulate in *Lastrea*, and aculeate in *Thelypteris* (Tryon and Lugardon, 1991).

Gametophyte development.—The gametophyte development for this species is here reported for the first time. Spores began to germinate eight days after sowing. The spore germination was of the *Vittaria*-type (Fig. 4A), and prothallial development was of the *Adiantum*-type (Fig. 4B) (Nayar and Kaur, 1971). After several divisions of a meristematic cell, a meristematic zone was formed, which was presented as an apical notch, on which few trichomes were present. The prothalli were bisexual although the antheridia and archegonia occurred on each prothallus asynchronously. Antheridia (Fig. 4C) were distributed over the dorsal surface or along the margin. Archegonia (Fig. 4D) were formed near the notch of the prothallium. Young sporophytes were covered with glands (Fig. 4E). In contrast, prothallial development of *Cyclosorus parasiticus* is of the *Drynaria*-type (Xie *et al.*, 2008), and young sporophytes were covered with more flexible hairs (Fig. 4F).

TABLE 2. Morphological and cytological comparison of related genera with *Mesopteris*.

Taxon	*Amphineuron*	*Cyclosorus*	*Dryopteris*	*Lastrea*	*Thelypteris*	*Mesopteris*
Frond venation	anastomosing	anastomosing	free	simple/forked	forked	free
covered	capitate hairs, with glands	hispid, with glands	scales, with long flexible hairs.	few soft hairs	acicular hairs	glabrous, with glands
texture	chartaceous	herbaceous/ chartaceous	chartaceous/ coriaceous	coriaceous	herbaceous	chartaceous
Sporangia	seldom glandular	glabrous/ hispid	few glands	glandular	glandular	glabrous
Indusium	indusiate	indusiate	indusiate	indusiate	indusiate	exindusiate
Spore ornamentation	tuberculate	lophate	verrucate/ alate	granulate	aculeate	cristae
Chromosome base number	$x = 36$	$x = 36$	$x = 41$	$x = 17$	$x = 35$	$x = 38$

Cytological observation.—The chromosome number for this species is here reported for the first time. *Mesopteris tonkinensis* had $2n=76$ chromosomes in mitotic root-tip cells (Figs. 5A–B), and $n=38$ pairs of chromosomes in meiotic cells (Figs. 5C–D). *Mesopteris* has the distinctive chromosome base number of $n=38$, which is different from $n=41$ of *Dryopteris*, $n=35$ of *Thelypteris*, $n=36$ of *Cyclosorus*, and $n=17$ of *Lastrea* (Love *et al.*, 1977). Sixty-four spores per sporangium were found, and homologous chromosomes paired normally, which suggests it is a sexually reproducing diploid (Wang *et al.*, 2011).

Table 2 provides a comparison of the characteristics of the related genera of *Amphineuron*, *Cyclosorus*, *Dryopteris*, *Lastrea*, and *Thelypteris*, with the genus *Mesopteris*.

All the results above demonstrate that *Mesopteris tonkinensis* is a distinctive species that possesses a distinctive suite of characters not found in related genera. From the data presented, we conclude that it is best to recognize this distinctive taxon in the monotypic genus *Mesopteris*.

Taxonomic Treatment

Mesopteris tonkinensis (C. Chr.) Ching, in Acta Phytotax. Sin. 16 (4):22. 1978. Basionym: *Dryopteris tonkinensis* C. Chr., in Bull. Mus. Paris. ser. 2. 6:102. 1934. Synonyms: *Thelypteris tonkinensis* (C. Chr.) Ching, in Bull. Fan. Mem. Inst. Biol., Bot. 6: 292. 1936; *Lastrea tonkinensis* (C. Chr.) Copeland, in Gen. Fil. 140. 1947; *Amphineuron tonkinense* (C. Chr.) Holttum, in Blumea. 23 (2):210. 1977. TYPE: VIETNAM. Liangshan: Agric Hanoi, 3415 (holotype: K!), not seen.

Additional Specimens Examined.—CHINA. Guangxi: Longzhou, live on limestone, 9 Apr 1991, Elev. 480 m, *Zhou 2846*; 12 Apr 1991, Elev. 500 m, *Zhou 2883*; Daxin, live on limestone, 24 March 1993, Elev. 300 m, *Zhou 3789* (Guangxi Agriculture University herbarium).

Distribution and Habitat.—This genus has a narrow range along the border region between China (Guangxi: Longzhou, Daxin, Napo) and Vietnam (Liangzhou). *Mesopteris tonkinensis* grows in roadsides or is lithophytic on limestone with mosses.

Acknowledgments

We would like to thank Prof. Cindy Q. Tang and Dr. Hong-Mei Liu for helpful suggestions of the manuscript. We sincerely thank reviewers and editors Warren D. Hauk and Tom A. Ranker of the American Fern Journal for providing valuable suggestions on this paper. This study was supported by the National Natural Science Foundation of China (Grant Nos. 31460049, 31200162, 31370240) and the Natural Science Foundation of Guangxi (Grant No. 2011GXNSFA018089).

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Rhopalotricha, a New Subgenus of the Fern Genus *Lastreopsis* (Dryopteridaceae)

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Abstract.—A new subgenus of *Lastreopsis*, subg. *Rhopalotricha*, is here described based on the results of our recent phylogenetic analyses. Its species differ from others in the genus by having a distinctive type of hair within the grooves of the rachises and costae adaxially. These are 1–3(–4)-celled and clavate, whereas the remainders of the species in the genus have hairs that are 3–12-celled and non-clavate. Subgenus *Rhopalotricha* also differs by having spores with broadly folded perispores with echinulate surfaces (vs. tuberculate or spiny, and with smooth surfaces). Subgenus *Rhopalotricha* occurs in the Neotropics, islands of the southwestern Pacific (Samoa, Fiji, and Vanuatu), New Zealand, and Australia. A key is provided to distinguish the species, each of which is treated with synonymy, description, geographic distribution by country, elevation ranges, and discussions. Lectotypes are also designated for *Dryopteris amplissima* var. *subeffusa*, *Aspidium macrum* Fée, and *Aspidium latissimum* Fée.

Key Words.—ferns, lastreopsids, taxonomy

Nested within the Dryopteridaceae (sensu Smith *et al.*, 2006) is a clade of about 150 species that we call the "lastreopsid ferns" (Labiak *et al.*, 2014a). The clade is pantropical with extensions into the temperate areas of the southern hemisphere. Members of the clade include *Lastreopsis* Ching, *Megalastrum* Holttum, *Parapolysticum* (Keyserl.) Ching, and *Rumohra* Raddi. The first genus is composed of two clades: a large clade of at least ten species, containing the type species of *Lastreopsis*, and a smaller clade – the *Lastreopsis amplissima* clade – whose relationship within the lastreopsids forms a polytomy with *Megalastrum*+*Rumohra* and the *Lastreopsis* s.s., clades (Labiak *et al.*, 2014a). The *L. amplissima* clade is composed of four species, three of which were included in our previous phylogenetic analysis; namely, *L. amplissima* (C. Presl) Tindale (South America), *L. hispida* (Sw.) Tindale (Australia and New Zealand), and *L. killipii* (Maxon) Tindale (Central and South America). One species not in our analyses, *L. davallioides* (Brack.) Tindale (Pacific Islands), is included here by us, in this taxonomic treatment, because it exhibits similar

morphology. The purpose of this paper is to describe the *L. amplissima* clade as a new subgenus of *Lastreopsis* and provide a synopsis of its species.

In her monograph of *Lastreopsis*, Tindale (1965) first pointed out the similarities among the four species, placed here in subg. *Rhopalotricha*. In fact, she separated these four species from their congeners in the first couplet of her key. The distinguishing characters used were rhizome scales with denticulate claw-like teeth, upper surfaces of the rachises provided with clavate-glandular hairs, suprabasal pinnae with the pinnules arranged catadromically, and spores that are finely echinulate. We confirm the usefulness and constancy of these characters except for pinnule arrangement, which we find variable in all four species.

In our phylogenetic analysis of lastreopsid ferns (Labiak *et al.*, 2014a), the three species (*L. amplissima, L. hispida*, and *L. killipii*) were resolved together as a strongly supported monophyletic group (Posterior Probability [PP] = 1.0, Maximum Likelihood Bootstrap [ML-BS]= 100%, and Maximum Parsimony Bootstrap [MP-BS] = 100%), but their relationship with the other clades within the lastreopsids remained ambiguous. In our Bayesian and Maximum Likelihood analyses it was recovered as sister to the rest of *Lastreopsis s.s* (PP = 0.91, and ML-BS = 66%). The Maximum Parsimony analysis, on the other hand, recovered it as sister to *Megalastrum+Rumohra* clade (MP-BS = 73%). Because of its strongly supported monophyly, morphological distinctiveness, and unique biogeographical history (Labiak *et al.*, 2014a), we believe it convenient to recognize the clade (*L. amplissima, L. hispida*, and *L. killipii*) as a new subgenus, for which we propose the name *Lastreopsis* subg. *Rhopalotricha*.

We also include a species not in our phylogenetic analysis, *Lastreopsis davallioides,* in subg. *Rhopalotricha,* because it shares the characters first pointed out by Tindale (1965). One of these characters—the echinulate perispore—can be compared to that of *L. amplissima, L. hispida,* and *L. killipii* (Fig. 1). The lamina dissection of *L. davallioides* greatly resembles that of *L. amplissima* (Fig. 2).

Materials and Methods

Herbarium specimens at B, MO, NY, P, UPCB, and VT were analyzed to write the key and comparative discussions. Geographic distributions were obtained primarily from Tindale (1965), as well as the list of synonyms and type information. The spores were imaged digitally at the New York Botanical Garden using a JEOL jSM-5410LV SEM. The images are also available at www.plantsystematics.org.

Results

Lastreopsis subg. **Rhopalotricha** Labiak & R. C. Moran, **subgen. nov.** Type: *Dryopteris killipii* C. Chr. & Maxon, Amer. Fern J. 18(1):4. 1928.

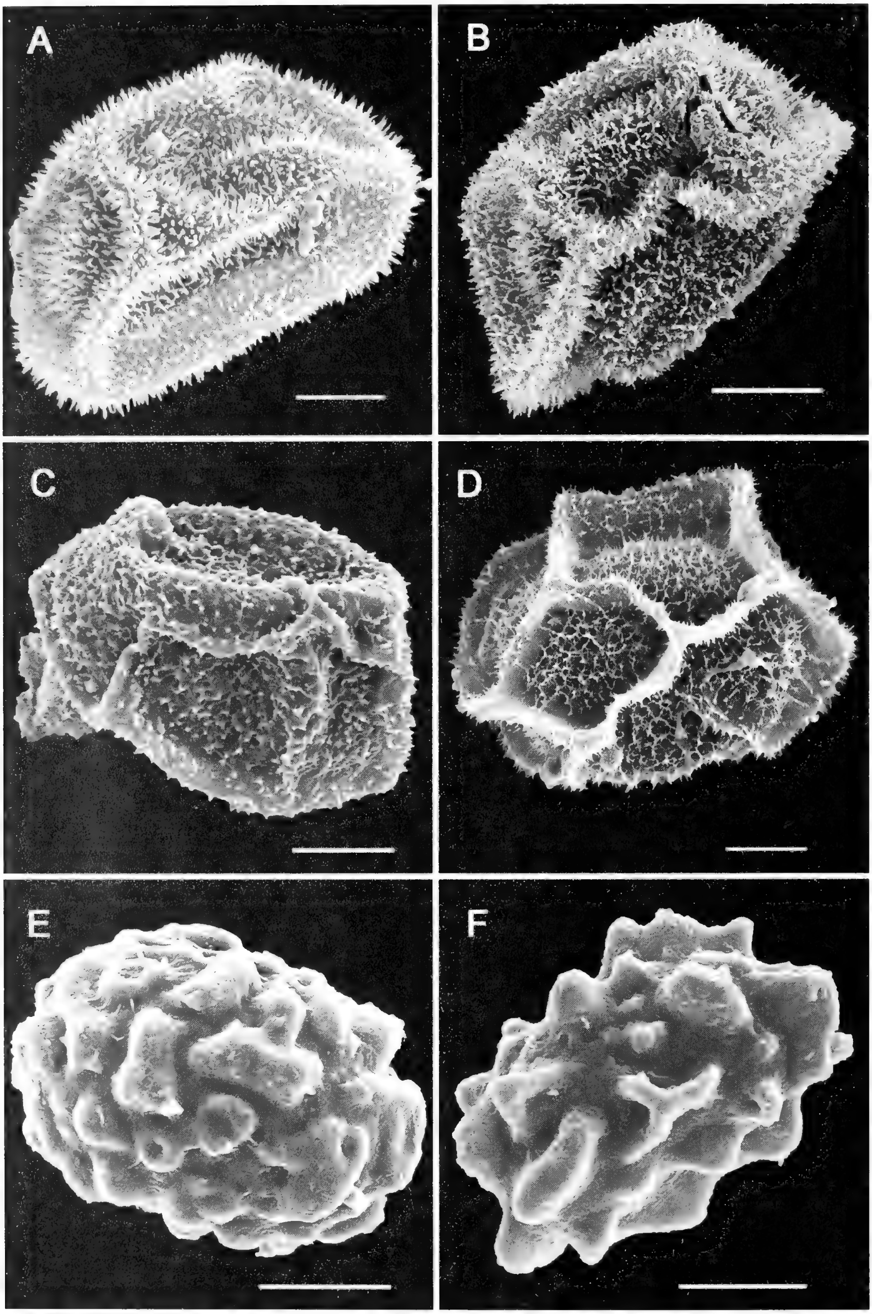

FIG. 1. Spores of *Lastreopsis* subg. *Rhopalotricha*, *Lastreopsis* subg. *Lastreopsis*, and *Parapolystichum*. (**A**) *Lastreopsis* (subg. *Rhopalotricha*) *killipii*. (**B**) *L.* (subg. *Rhopalotricha*) *amplissima*. (**C**) *L.* (subg. *Rhopalotricha*) *davallioides*. (**D**) *L.* (subg. *Rhopalotricha*) *hispida*. (**E**) *L.* (subg. *Lastreopsis*) *marginans*. (**F**) *Parapolystichum confine*. Scale bars = 10 micrometers. (A from Venezuela, *Beitel*

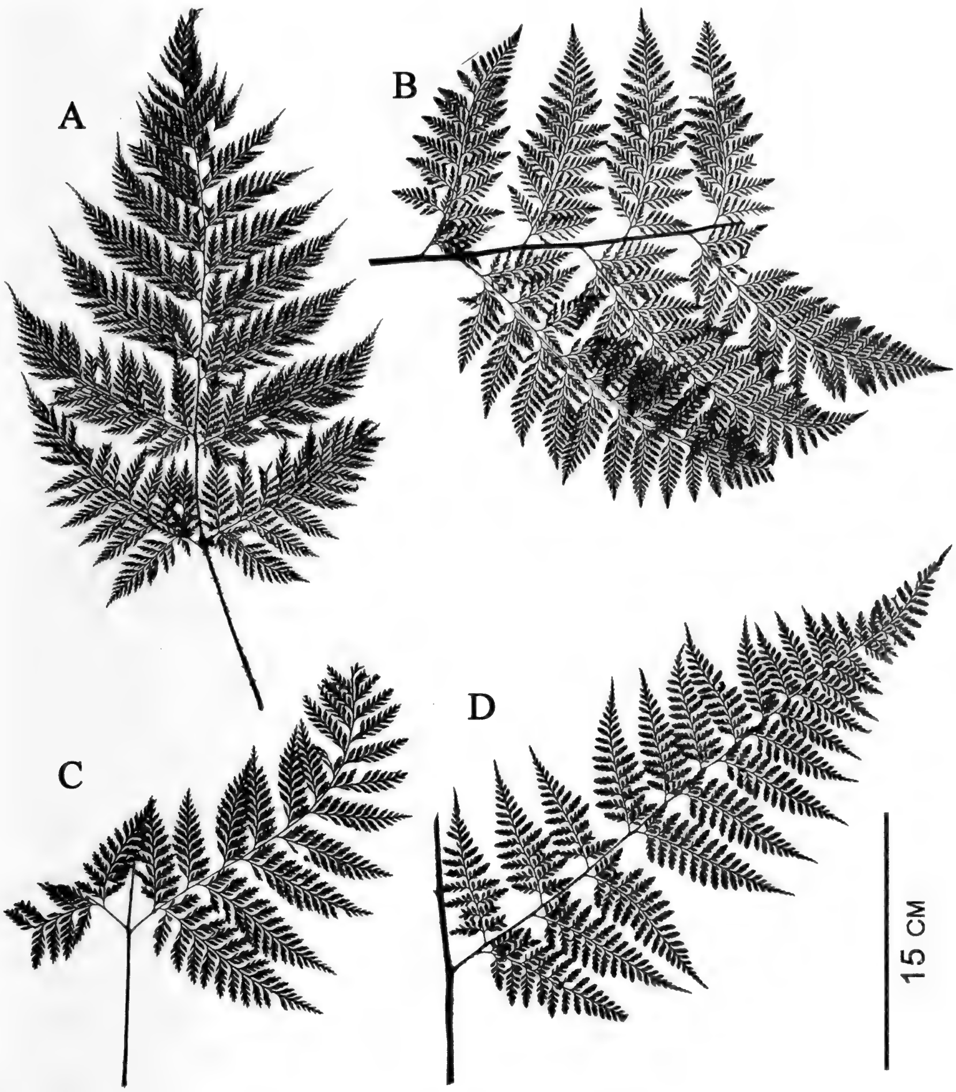

FIG. 2. Silhouettes of the laminae and pinnae of *Lastreopsis* subg. *Rhopalotricha*. (**A**) *L. hispida*, complete leaf. (**B**) *L. amplissima*, basal pinna. (**C**) *L. davallioides*, basal pinna. (**D**) *L. killipii*, basal pinna. (A from New Zealand, *collector unknown s.n.*, NY; B from Brazil, *Webb s.n.*, NY; C from Samoa, *Vanpaul 336*, NY; D from Venezuela, *Beitel 85141*, NY).

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85147, NY; B from Paraguay, *Hahn 756*, NY; C from Fiji, *Smith 275*, NY; D from Australia, *Coveny & Haegi 9942*, NY; E from Cuba, *Howard 5233*, NY). Figures A, C, D, and E originally published in Labiak et al., 2014a).

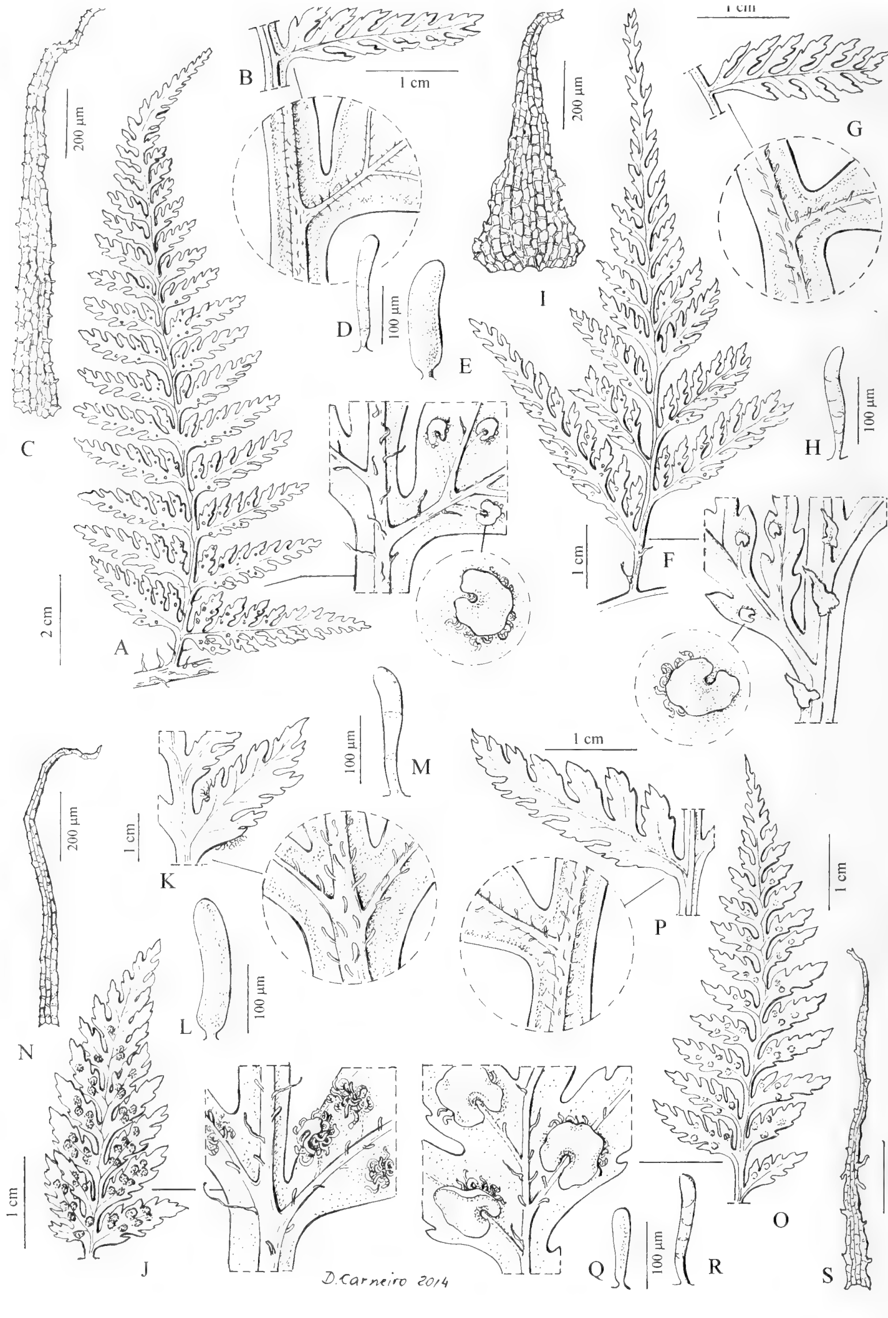

FIG. 3. **(A–E)** *Lastreopsis amplissima.* **(A)** Basal pinna, showing a detail of the abaxial surface and the sori. **(B)** Adaxial side of a pinnule, showing the hairs on the axes. **(C)** Scale from the rachis. **(D)** Club-shaped hair from the adaxial side. **(E)** Glandular hair from the abaxial side of the lamina. **(F–I).** *L. davallioides.* **(F)** Basal pinna, showing a detail of the abaxial surface and the sori. **(G)** Adaxial side of a pinnule, showing the hairs on the axes. **(H)** Club-shaped hair from the adaxial side. **(I)** Scale from the rachis. **(J–N)** *L. hispida.* **(J)** Basal pinna, showing a detail of the abaxial surface and

Plants terrestrial; *rhizomes* short, stout, ascending, scaly, the scales denticulate; *leaves* up to 2 m long; *petioles* about as long as the laminae; *laminae* 3- to 5-pinnate-pinnatifid, deltate or pentagonal to broadly lanceolate, gradually tapered to a pinnatifid apex, buds on rachises absent; *basal pinnae* inequilateral prolonged on the basiscopic side; *suprabasal pinnae* equilateral or nearly so; *pinnules* of suprabasal pinnae anadromic; *rachises, costae, and costules* with a thickened marginal ridge surcurrent on the segments of the next higher order (i.e., "*Lastreopsis*-type" rachis-costa architecture), adaxially pubescent, the hairs 0.1–0.3 mm long, 1–3(–4)-celled, clavate, usually reddish brown, the apical cell sometimes swollen, apparently glandular and often darker than the others; *veins* free, ending at or very near the margins, abaxially glandular, the glands ca. 0.1 mm long, thick, oblong-cylindrical, shiny, yellow, orange or reddish, often appressed; *sori* round, indusiate, the indusia reniform, light brown to blackish, attached by a sinus; *sporangia stalks* glabrous or with a colored gland, the gland oblong-cylindrical; *annulus* of 14–17 indurated cells; *spores* monolete, achlorophyllous, the perispores finely echinulate, folded, the folds long, uninterrupted. x=41 (Brownlie, 1958).

Subgenus *Rhopalotricha* is characterized by denticulate rhizome scales, whereas those of subg. *Lastreopsis* are entire or nearly so. The rachises, costae, and costules are pubescent adaxially with 1–3(–4)-celled, clavate-glandular hairs, the apical cell of which is often darker than the others. This character is referenced in the name of the new genus (from the Greek, *rhopalo*, club, and *tricho*, hair) (Fig. 3). In contrast, species of subg. *Lastreopsis* and the genus *Parapolystichum* have slender, non-clavate hairs, composed of 3–12 cells. Subgenus *Rhopalotricha* has broadly folded perispores with the folds uninterrupted and finely echinulate surfaces. In contrast, *Lastreopsis* and *Parapolystichum* have perispores with folds interrupted to form a tuberculate pattern, and the surfaces are smooth or nearly so (Fig. 1; see this character optimized onto a phylogenetic tree depicting relationships of lastreopsid ferns in Fig. 6, Labiak *et al.*, 2014a). Unlike many species of *Parapolystichum* (e.g., *P. effusum* (Sw.) Ching and *P. excultum* (Mett.) Labiak, Sundue & R. C. Moran), subg. *Rhopalotricha* lacks buds on rachises (see this character optimized onto a phylogenetic tree depicting relationships of lastreopsid ferns in Fig. 5, Labiak *et al.*, 2014a).

Regardless of whether subg. *Rhopalotricha* will be confirmed as sister to subg. *Lastreopsis* or to *Rumohra*+*Megalastrum*, *Rhopalotricha* could be alternatively recognized at the rank of genus, since its monophyly is strongly

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the sori. **(K)** Adaxial side of a pinnule, showing the hairs on the axes. **(L)** Glandular hair from the abaxial side of the lamina. **(M)** Club-shaped hair from the adaxial side. **(N)** Scale from the rachis. **(O–S)** *L. killipii*. **(O)** Basal pinna, showing a detail of the abaxial surface and the sori. **(P)** Adaxial side of a pinnule, showing the hairs on the axes. **(Q)** Glandular hair from the abaxial side of the lamina. **(R)** Club-shaped hair from the adaxial side. **(S)** Scale from the rachis. (A–E from Brazil, *Labiak et al. 4294*, UPCB; F–I from Fiji, *A.C. Smith 275*, NY; J–N from New Zealand, *Pichi-Sermolli 6240*, NY; O–S from Bolivia, *Altamirano 952*, NY).

supported. We prefer to recognize this monophyletic group in the rank of subgenus because of the morphological similarity with *Lastreopsis* subg. *Lastreopsis* in rhizome habit, lamina division, and sori. Using this rank fosters nomenclatural stability because the species of subg. *Rhopalotricha* have been classified in *Lastreopsis* by all pteridologists since Tindale's monograph of the group (1965).

The following key can be used to distinguish the new subgenus from *Lastreopsis* subg. *Lastreopsis* (corresponding to the clade *Lastreopsis* s.s., as termed by Labiak *et al.,* 2014a) and the morphologically similar *Parapolystichum,* in which many species formerly classified in *Lastreopsis* are now placed (Labiak *et al.*, 2014b). *Lastreopsis* and *Parapolystichum* cannot be consistently distinguished morphologically, and therefore only tendencies are given in the key.

Key to the Subg. *Rhopalotricha*, Subg. *Lastreopsis*, and *Parapolystichum*

1. Hairs on the adaxial surfaces of the rachises and costae clavate, 1–3(–4)-celled, the apical cell often enlarged and apparently glandular; rhizome scales denticulate; perispores with uninterrupted folds and finely echinulate surfaces. . . . . *Lastreopsis* subg. *Rhopalotricha*
1. Hairs on the adaxial surfaces of the rachises and costae slender throughout, 3–12-celled, the apical cell not enlarged or glandular; rhizome scales entire or nearly so; perispores tuberculate or coarsely spiny, the surfaces of the tubercles or of the coarse spines otherwise smooth
   2. Rhizomes short-creeping or erect; buds on rachises present or absent; plants from Africa, Islands of Western Indian Ocean, Australasia, and Neotropics . . . . *Parapolystichum*
   2. Rhizomes long-creeping or ascending; buds on rachises absent; plants from Australasia and Neotropics . . . . *Lastreopsis* subg. *Lastreopsis*

The species of subg. *Rhopalotricha* may be distinguished by the following key. The synonymy, type information, and distribution data after each species largely follows that given by Tindale (1965).

Key to the Species of *Lastreopsis* subg. *Rhopalotricha*

1. Leaves 0.3–1.0 m long, rachis scales thick, subulate, terete, widely spreading; abaxial surfaces of rachises and costae with hairs 0.2–0.5 mm long, 3–5-celled, spreading; Australia and New Zealand. . . . . *L. hispida*
1. Leaves 1–2 m long, rachis scales thin, flat, loosely spreading to appressed; abaxial surfaces of rachises and costae without hairs; South Pacific Islands, Central and South America
   2. Rhizomes long-creeping; rachis scales 0.5–1.5 mm long, lanceolate, entire; Samoa, Fiji, Vanuatu . . . . *L. davallioides*
   2. Rhizomes short-creeping to ascending; rachis scales 1–5 mm long, linear-lanceolate, erose to denticulate; Central and South America
      3. Petioles, rachises, and costae densely scaly; veins abaxially glandular; penultimate and ultimate segments typically spreading; Costa Rica, Panama, s. Venezuela, Colombia, Ecuador, and Peru. . . . . *L. killipii*
      3. Petioles, rachises, and costae sparsely scaly; veins abaxially eglandular or sparsely and inconspicuously glandular; penultimate and ultimate segments often ascending; s. Venezuela, s. Brazil, Paraguay, and Bolivia . . . . *L. amplissima*

Lastreopsis* (subg. *Rhopalotricha*) *amplissima (C. Presl) Tindale, Vict. Naturalist 73:185. 1957. *Polystichum amplissimum* C. Presl, Tent. Pterid.

84. 1836, *nom. nud.*; Epim. Bot. 58. 1849 [1851]. *Aspidium amplissimum* (C. Presl) Mett., Abh. Senckenberg. Naturf. Ges. 2(2):352. 1858. *Nephrodium amplissimum* (C. Presl) Hook., Sp. Fil. 4:145. 1862. *Dryopteris amplissima* (C. Presl) O. Kuntze, Rev. Gen. Pl. 2:812. 1891. *Ctenitis amplissima* (C. Presl) Copel., Gen. Fil. 124. 1947. *Rumohra amplissima* (C. Presl) Ching., Sinensia 5:35. 1934. *Polystichopsis amplissima* (C. Presl) Abbiatti, Revista Mus. La Plata, Secc. Bot. 37:19. 1958. TYPE: BRAZIL. [probably Rio de Janeiro or São Paulo]: exact locality unknown, s.d. [ca. 1822], *F. Sellow & H. K. Beyrich* (Holotype: PR n.v.; Isotype: B n.v.). Figs. 1B, 2B, and 3A–E.

Aspidium macrum Fée, Crypt. Vasc. Brésil 1:141–142, t. 48, f. 1. 1869. TYPE: BRAZIL, Habitat in Brasilia fluminensi, *A. Glaziou 2390* (Lectotype, here designated: P! P02433921; isolectotype B!).

Aspidium latissimum Fée, Crypt. Vasc. Brésil 1:142, t. 48, f. 2. 1869. TYPE: BRAZIL, Habitat in Brasilia fluminensi, *A. Glaziou 979* (Lectotype, here designated: P! P00642655 Isolectotypes: P! P02433922, P00642654, P02433923).

Dryopteris amplissima (C. Presl) O. Kuntze var. *subeffusa* C. Chr., Kongel. Danske Vidensk. Selsk. Skr., Naturvidensk. Afd., Ser. 8. 6:112. 1920. TYPE: VENEZUELA. Amazonas: Mt. Roraima, s.d., *R. Schomburgk 1151* (Lectotype, here designated: P! P00642656).

Rhizomes short, thick, erect or ascending; *leaves* 1–2 m long; *petioles* slightly longer than the laminae, densely scaly toward the base and becoming less so distally, the scales lanceolate, appressed to slightly spreading, reddish-brown, thin, sharply toothed, the teeth often curved; *laminae* 0.5–1.0 × 0.7–0.9 m, 4-pinnate to 5-pinnate-pinnatifid, deltate-ovate, anadromous throughout; *rachises, costae, and costules* sparsely scaly abaxialy, the scales 1–3 mm long, linear-lanceolate, slightly tortuous, dark brown; *basal pinnae* 30–45 × 25–30 cm, ovate-oblong to triangular-oblong; *ultimate segments* 5–8 × 2–3 mm, obtuse to acute, often ascending and sometimes strongly so; *tissue between the veins* glabrous on both surfaces; *sporangial stalks* glabrous; *indusia* 1.0–2.2 mm long, glabrous.

Distribution.—southern Venezuela, Peru, Bolivia, Brazil, Argentina, and Paraguay; 150–1460 m.

This species greatly resembles *Lastreopsis davallioides* in dissection of the laminae but differs by ciliate scales on the rachis (the scales are entire in *L. davallioides*).

Lastreopsis* (subg. *Rhopalotricha*) *davallioides (Brack.) Tindale, Vict. Naturalist 73:184. 1957. *Lastrea davallioides* Brack., U.S. Expl. Exped., Filic. 16:202. 1854. *Dryopteris davallioides* (Brack.) O. Kuntze, Rev. Gen. Pl. 2:812. 1891. *Parapolystichum davallioides* (Brack.) Ching, Sunyatsenia 5:239. 1940. *Ctenitis davallioides* (C. Presl.) Copel., J. Arn. Arbor. 30:437. 1949. TYPE: TAHITI [but see below section Distribution]. "No. 19, U.S.

South Pacific Exploring Expedition, under the command of Capt. Wilkes, 1838–42'' (Holotype: US 56405-56406, images seen). Figs. 1C, 2C, and 3F–I.

Dryopteris microtricha Copel., Bish. Mus. Bull. 59:10, 44. 1929. *Ctenitis microtricha* (Copel.) Copel., Gen. Fil. 124. 1947. TYPE: FIJI. Viti Levu, Voma Mountain, near the summit, 900 m, 1927, *J. W. Gillespie 2742* (Holotype: BISH 1000481-1000482, images seen; Isotype UC 353779, image seen).

Rhizomes long-creeping; *leaves* 1.0–1.5 m long; *petioles* slightly longer than the laminae, densely scaly toward the base and becoming less so distally, the scales loosely spreading, linear, dark brown to golden brown, thin, sharply toothed, the teeth often recurved; *laminae* 0.3–0.6 × 0.2–0.3 m, 4-pinnate-pinnatifid to 5-pinnate, pentagonal, anadromous throughout; *rachises, costae, and costules* sparsely scaly abaxially, the scales 0.5–1.5 mm long, lanceolate, dark brown, entire; *basal pinnae* 20–40 × 14–25 cm, ovate-oblong to triangular-oblong; *ultimate segments* 4–7 × 1–2 mm long, obtuse to acute, sometimes strongly ascending and slightly falcate; *tissue between the veins* adaxially glabrous or nearly so, sparsely glandular abaxially, the glands minute, thick shiny, oblong, cylindrical, orange or yellowish, often appressed; *veins* glandular, the glands ca. 0.1 mm long, oblong, yellow or orange; *sporangia stalks* often with a colored glandular hair; *indusia* ca. 1 mm long, glabrous or with a few glandular hairs toward the center.

Distribution.—Samoa, Fiji, Vanuatu; 600–900 m. Although noted from Tahiti, the type specimen is likely from other islands in the Southern Pacific, given that the species has never been observed in Tahiti, and no collection is known from French Polynesia (including the island of Tahiti) in any of the herbaria relevant for these territories (J. Florence, pers. com.).

Christensen (1943) noted that the yellowish (sometimes orange) glands on the veins abaxially are so dense that the veins appear under magnification as yellow stripes. *Lastreopsis davallioides* greatly resembles *L. amplissima* in lamina size and cutting but can be distinguished by entire and lanceolate scales on the lamina (vs. erose and linear-lanceolate). If these two species are sister taxa as morphology suggests, it would be a remarkable example of a floristic relationship between the southwest Pacific Islands and southern South America.

Lastreopsis* (subg. *Rhopalotricha*) *hispida (Sw.) Tindale, Vict. Naturalist 73:183. 1957. *Aspidium hispidum* Sw., J. Bot. (Schrader) 1800(2):39. 1801. *Polystichum hispidum* (Sw.) J. Sm., J. Bot. (Hooker) 4:195. 1841. *Lastrea hispida* (Sw.) T. Moore & Houlston, Garden Mag. Bot. 3:88. 1851. *Nephrodium hispidum* (Sw.) Hook., Sp. Fil. 4:150. 1862. *Dryopteris hispida* (Sw.) O. Kuntze, Rev. Gen. Pl. 2:813. 1891. *Rumohra hispida* (Sw.) Copel., Gen. Fil. 114. 1947. TYPE: NEW ZEALAND. s.d., *Forster s.n.* (Lectotype, designated by Tindale (1965): S P6231, image seen; isolectotypes: BM 001048428, image seen, GOET n.v.). Figs. 1D, 2A, and 3J–N.

Rhizomes long-creeping; *leaves* 0.3–1.0 m long; *petioles* ca. as long as the laminae, densely scaly, the scales subulate, terete, thick, widely spreading, reddish-brown, entire; *laminae* 0.5–1.0 × 0.7–0.9 m, 3–4-pinnate, deltate-ovate, the basal pinnae catadromous, suprabasal pinnae anadromous; *rachises, costae, and costules* pubescent and conspicuously scaly abaxially, the hairs 0.2–0.5 mm long, 3–5-celled, spreading, the scales like those of the petiole; *basal pinnae* 6–13 × 5–10 cm, ovate-oblong to triangular-oblong; *ultimate segments* 2–12 × 1.0–2.5 mm, narrowly lanceolate, slightly falcate, acuminate; *tissue between the veins* on both surfaces glabrous or with very sparse glands; *veins* glandular on both surfaces, more so abaxially, the glands ca. 0.1 mm long, oblong-cylindrical, colored, appressed; *sporangial stalks* glandular, the glands colored; *indusia* 1.0–2.2 mm long, with colored glands on the margins.

Distribution.—southeastern Australia, New Zealand, and Chatham Islands; 0–800 m.

Lastreopsis hispida differs from its congeners most conspicuously by leaves beset with dark, patent, bristle-like scales. Other distinguishing characteristics are given in the key. This is the only species of the subgenus that has had its chromosomes counted, with the result of n=41 (Brownlie, 1958).

In her description of this species, Tindale (1965) stated that laminae were "catadromous at the base, anadromous above." We find, however, the pinnules may be arranged either catadromously or anadromously, especially in the suprabasal pinnae.

Lastreopsis* (subg. *Rhopalotricha*) *killipii (C. Chr. & Maxon) Tindale, Vict. Naturalist 73:185. 1957. *Dryopteris killipii* C. Chr. & Maxon, Amer. Fern J. 18:4. 1928. *Parapolystichum killipii* (C. Chr. & Maxon) Ching, Sunyatsenia 5239. 1940. TYPE: PANAMA. Chiriquí: in humid forest of the Río Caldera watershed, W of El Boquete, 1900 m, 17–19 Feb 1917, *E. P. Killip 5360* (Holotype: US 01100899, image seen; Isotype: MO! 122596). Figs. 1A, 2D, and 3O–S.

Rhizomes short, thick, ascending; *leaves* 1–2 m long; *petioles* slightly longer than the laminae, densely scaly throughout, the scales loosely spreading, lanceolate, reddish-brown, thin, flat, appressed, denticulate; *laminae* 0.5–1.0 × 0.7–0.9 m, 4-pinnate-pinnatifid, deltate to broadly ovate; *rachises, costae, and costules* usually densely scaly abaxially, the scales 1–5 mm long, lanceolate, flat, reddish brown, denticulate; *basal pinnae* 30–45 × 20–30 cm, ovate-oblong to triangular-oblong; *ultimate segments* 4–6 × 2.5–4 mm, obtuse; *tissue between the veins* adaxially glabrous, glandular abaxially, the glands minute, thick shiny, oblong-cylindrical, yellowish, appressed; *sporangial stalks* glabrous; *indusia* 1.0–2.0 mm long, glabrous.

Distribution.—Costa Rica, Panama, Colombia, s. Venezuela, Ecuador, and Peru; 1300–2360 m.

Lastreopsis killipii is characterized by densely scaly petioles, rachises, and costae, especially abaxially (Fig. 3, O–R).

Acknowledgments

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Reproductive Biology of *Aglaomorpha cornucopia* (Copel.) M.C. Roos, a Rare and Endemic Fern from the Philippines

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Abstract.—*Aglaomorpha cornucopia* (Copel.) M.C. Roos is an endemic and rare epiphytic fern from the Philippines. *Ex situ* germplasm storage and growth are important complementary tools for conserving this rare fern. This study was conducted to document the reproductive biology of this species. Mature sporophylls of *A. cornucopia* were collected in May, 2012 from Mt. Apo, the Philippines. Each sporangium bore 64 yellow, monolete spores. The average spore size was 49.3 ± 3.7 µm. Fresh spores germinated 100% within one week of sowing (mean germination time (MGT) <1 week). Air-dried mature spores remained completely viable even after one year of storage at 3°C, although mean germination time was somewhat delayed (MGT=1.4 weeks). Spore germination was of the *Vittaria*-type, whereas gametophyte development was of the *Drynaria*-type. Adult gametophytes were cordiform-annual and gametangia were of the leptosporangiate type. Unicellular papillate hairs appeared on marginal, dorsal, and ventral surfaces of the gametophytes. Gametophytes first produced antheridia and archegonia after seven weeks of culture. Gametophytes began to sexually produce sporophytes after 13 weeks of culture. The rate of sporophyte production reached 64% after 26 weeks culture. Results of this study suggest that cold temperature spore storage and *in vitro* culture offer reliable techniques for conserving this rare fern.

Key Words.—gametophyte, sexual expression, spore viability, young sporophyte

Aglaomorpha Schott (Polypodiaceae) is a genus of large-sized ferns of tropical Asia, comprising about 13–14 species (Janssen and Schneider, 2005; Roos, 1986). Four species of *Aglaomorpha* are native to the Philippines, including *A. cornucopia* (Copel.) M.C. Roos, *A. pilosa* (J. Sm.) Copel., *A. meyeniana* Scott, and *A. splendens* (J. Sm.) Copel. (Copeland, 1960). *Aglaomorpha cornucopia*, once classified as *Thayeria cornucopia* Copeland (Copeland, 1960), is an endemic epiphyte in the Philippines. It is distributed in the mountains of Mindanao and Luzon (Barcelona, 2005; Copeland, 1960)

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and regarded as a rare species due to its limited distribution (Amoroso, 1987; Amoroso *et al.*, 2011; Barcelona, 2005; Copeland, 1960).

Although *in situ* conservation should be the primary focus for conserving this fern, germplasm storage and *in vitro* culture are also important complementary conservation tools (Pence, 2004). Fern spore banking has been implemented for both research and germplasm storage (Huang *et al.*, 2003b; Ko *et al.*, 2006; Lloyd and Klekowski, 1970; Pence, 2000) and can preserve thousands of genotypes in much less space and lower cost than is needed to grow one plant (Pence, 2008). The use of cold storage temperatures may expand spore longevity in many ferns (Pence, 2008). In addition to spore banking, gametophytes and sporophytes also offer opportunities for *ex situ* conservation by *in vitro* cultures (Chao *et al.*, 2010; Chiou *et al.*, 2006; Chou *et al.*, 2007; Huang *et al.*, 2001; Pence, 2004, 2008).

The goal of this study was to explore the reproductive biology of the endangered *Aglaomorpha cornucopia* and to evaluate the efficacy of cold storage as a method for prolonging spore longevity. The viability of spores of *A. cornucopia* that had been stored for one year was monitored. Gametophyte development and reproductive biology were observed.

Materials and Methods

Two sporophylls of *Aglaomorpha cornucopia* were collected from one individual on May 2012 in the Energy Development Corporation (EDC) forest, Mt. Apo, Mindanao, the Philippines (N 7° 0′ 53″ E 125° 13′ 14″; elev. 1344m).

The sporophylls were air-dried in the laboratory for seven days to release spores. The frequency of normal spores and the length of the longest axis of 100 randomly sampled spores were measured. Spores were stored in a refrigerator at 3°C. Voucher specimens (*Kuo 2792, Amoroso et al. CMUH0007821*) were deposited in the herbaria of the Taiwan Forestry Research Institute (TAIF) and of Central Mindanao University (CMUH).

Fresh spores and one-year-old spores, which had been stored at 3°C, were sown onto four separate membrane filters (Pall Supor®-450, Michigan, USA) in each of four plastic boxes (PHYTATRAY II™ No. P5929, Sigma, USA) under LED white fluorescent illumination of 6.3±0.3 μmole m^{-2} s^{-1} for 10 h d^{-1} (LI-COR, light meter, LI-250A). The daily temperature ranged from 20–28°C. Humidity was monitored to avoid desiccation of the cultures. To count the germination rate and the mean germination time (MGT), 100 randomly sampled spores (25 spores per membrane filter) were observed at 1-week intervals after sowing for a total of 4 weeks (until the germination rate did not increase). The MGT was calculated based on the equation of Ellis and Roberts (1981):

$$\mathrm{MGT} = \sum(\mathrm{fx}) \Big/ \sum \mathrm{x}$$

where x is the number of spores germinated in week f, and f is the number of weeks counted from the beginning of culture.

To observe the development and sexual expression of gametophytes, fresh spores were sown into the four boxes (PHYTATRAY II™ No. P5929, Sigma, USA) filled with medium (vermiculite:peat:perlite, 4:4:2). The densities were 180–220 spores cm^{-2}, with culture conditions as above. One hundred randomly sampled gametophytes (25 per box) were removed (and not returned) and their sexual expression, age, and size were recorded during weeks 6, 7, 9, 11, and 13 after spore sowing. The percentages of gametophytes bearing sporophytes were recorded until the percentage remained stable. The mean sporophyte production time (MSPT) was calculated similar to the MGT, as:

$$\mathrm{MSPT} = \sum(\mathrm{fs}) \Big/ \sum \mathrm{s}$$

where s is the number of sporophytes produced in week f, and f is the number of weeks counted from the beginning of culture.

To assess sexual reproduction, sporophyte formation was observed by making paraffin sections. Three gametophytes, which bore sporophytes, were fixed in FPGA (Formalin:Propionic acid:Glycerol:95% Ethanol:distilled water = 1:1:3:7:8) overnight, dehydrated in an alcohol-TBA (*tert*-butanol) series for one day, infiltrated with paraffin (Paraplast Plus*, Kendall) at 60°C for one day, embedded in paraffin, and sectioned (10 μm thick serial sagittal sections of sporophyte) with a rotary microtome (RM2255, Leica).

Relative DNA levels between gametophytes and sporophytes were estimated to assess if sporophytes were produced sexually or apogamically. A total of about 5 cm^2 of gametophyte tissue from a total of 10 gametophytes and a total of 5 cm^2 from 5 fronds of one sporophyte were collected, respectively, minced in a chopping buffer (Epics-XL, Beckman) on ice, filtered through nylon mesh (20 μm, Peak Technology, Taiwan), cultured at 37°C for 15 minutes, and stained with 2.5% propidium iodide solution at 4°C in the dark for 15 minutes. The histogram peaks of genome size determined from the flow cytometer (FACScan, BD Technologies) were used to estimate relative ploidy levels of gametophytes and sporophytes (Cousin *et al.*, 2009).

Morphological observations were made with light microscopes (Leica, Wild M8; Leitz, Dialux 20) or a tabletop scanning electron microscope (SEM) (Hitachi, TM3000).

Results

There were 64 spores per sporangium. All fresh mature spores of *Aglaomorpha cornucopia* were yellow, bilateral, and monolete, with simple laesura. The average length of the spores was 49.3±3.7 μm. The perine surface was tuberculate to ornate, or somewhat vermiculate on the proximal side. The marginal processes were flat to rounded-verrucate, ornamented with echinate and papillate projections and the exospore was smooth (Fig. 1).

Both fresh and one-year-old spores attained 100% germination; however, germination time differed. Fresh spores germinated within one week after they

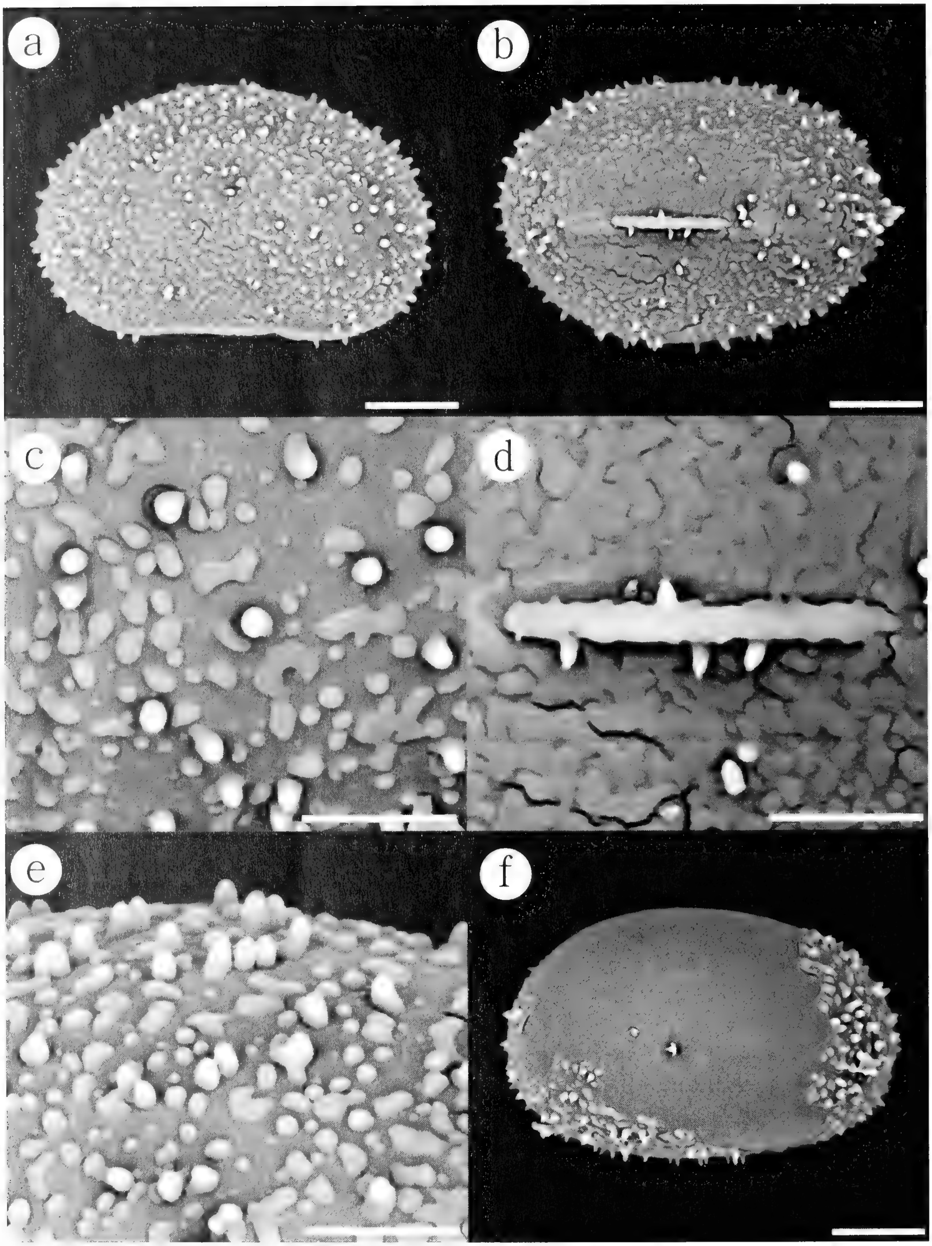

FIG. 1. SEM image of *Aglaomorpha cornucopia* spore. a: lateral view. b: proximal view. c: tuberculate to ornate perine sculpture. d: laesura and vermiculate perine sculpture at proximal end. e: flat to rounded verrucate marginal processes ornamented with echinate and papillate projections on perine. f: abraded spore, showing the plain exospore below. a, b, f: bars = 10 μm; c, d, e: bars = 5 μm.

were sown. The mean germination time (MGT) was less than 1.0 week. After one-year cold storage (3°C), spore germination was somewhat delayed compared to that of fresh spores with a MGT of 1.4 weeks, which was not significantly different from the former (*t*-test, $p>0.05$).

Following spore germination, the spore wall ruptured from the laesura on the proximal surface (Fig. 2a). A rhizoidal cell and a basal cell were formed by the first cell division parallel to the equatorial plane of the spore (Fig. 2b). Then the first rhizoid elongated (Fig. 2c), and a uniseriate filament was formed parallel to the polar axis of the spore by a series of transverse divisions (Figs. 2d–f).

When gametophytes were 3 to 5 cells long, filament growth was terminated. The middle cells divided parallel to the axis of the filament. Usually all the filament cells except the apical cell and basal cell divided longitudinally. Next, broad spathulate plates were formed (Figs. 2g–j). A wedge-shaped, meristematic cell formed in the apical region when the spathulate gametophytes were ca. 5 cells wide (Fig. 2j). The apical meristematic cell underwent repeated oblique divisions until it was replaced by a pluricellular meristem, which divided actively and formed an apical notch (Figs. 2k–m). Eventually, symmetrical (or nearly) cordate gametophytes were formed (Figs. 2n–o). Unicellular papillate hairs were produced on the margins when gametophytes were 3–5 cell wide, and were scattered on surfaces and margins with age.

When gametophytes were about 1 mm wide, a cushion with >one cell layers formed behind the meristem. The wings of young gametophytes were one cell thick and were usually flat, but became more curved, ruffled, and irregularly crenate with age.

Gametophytes first produced antheridia during early heart-shaped stages, when they were more than 0.5 mm wide, after 7 weeks of culture. However, some filamentous or spathulate male gametophytes less than 0.5 mm wide were found after 9 weeks. Antheridia appeared on the ventral surfaces and/or margins of the gametophytes where they were only one cell layer thick. They often intermingled with rhizoids (Fig. 3a). The hemispherical to subglobose antheridium wall was composed of a basal cell, a lower ring cell, an upper ring cell, a crescent-shaped cell, and an elliptical opercular cell (Fig. 3b). When mature antheridia were watered, the opercular cell and crescent-shaped cell were shed, and 32 spermatozoids were released from each antheridium.

Archegonia formed on the ventral surfaces of cushions approaching the notch (Fig. 4a). They did not appear until gametophytes were about 2 mm wide, after 7 weeks of culture. The necks of archegonia were composed of 4 tiers of cells, with 4 cells per tier (Fig. 4b).

Hermaphroditic gametophytes formed at ca. 4 weeks after male and female gametophytes were formed. The sizes of male gametophytes were significantly smaller than those of female and hermaphroditic ones (*t*-test, $p<0.05$). Female and hermaphroditic gametophytes were similar in size (Table 1). In most hermaphroditic gametophytes, antheridia were usually produced prior to cushion formation while the archegonia occurred only after the cushion

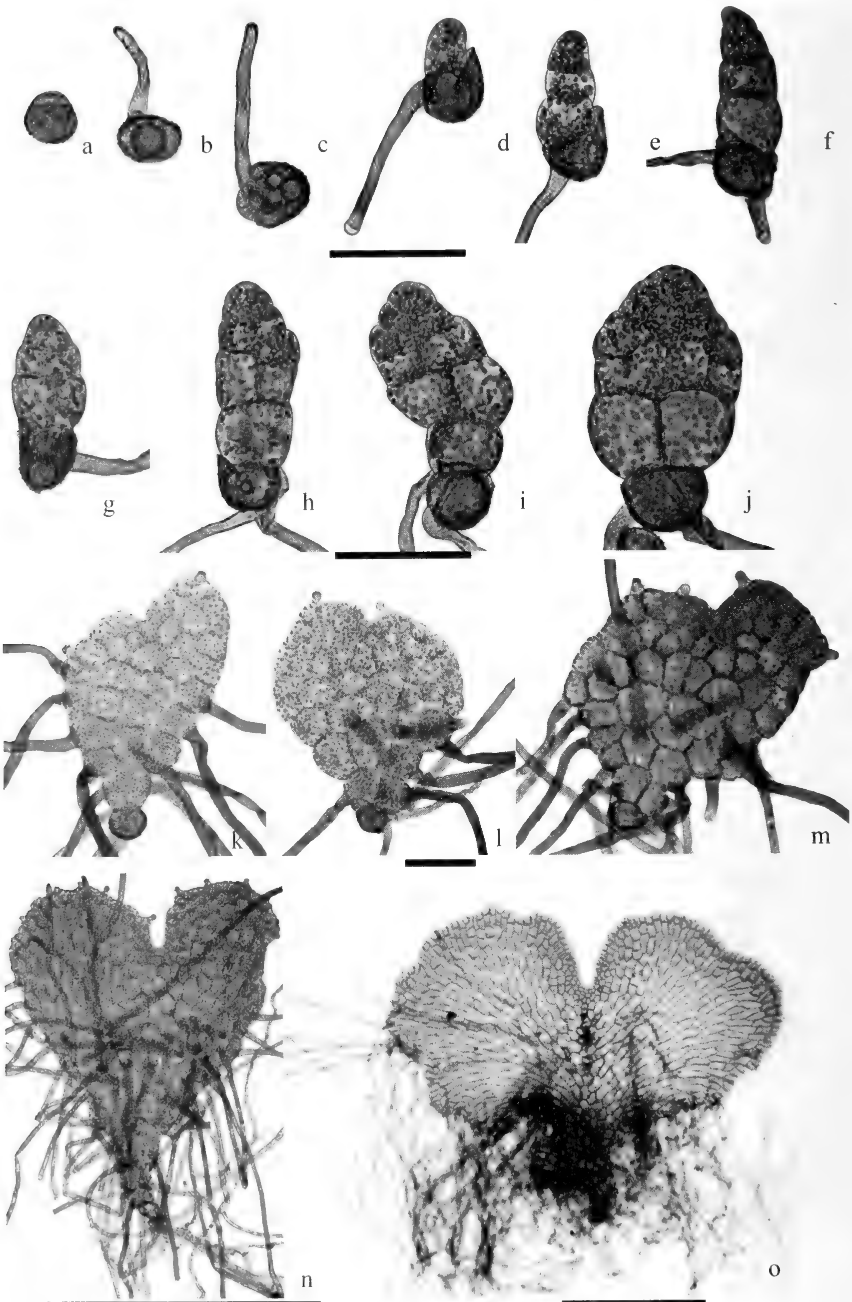

FIG. 2. Morphology of the gametophytes of *Aglaomorpha cornucopia*. a–b: rupture of spore, upper side denotes the position of the proximal surface. c: elongation of the first rhizoid. c–f: uniseriate gametophyte. g–j: spathulate gametophyte. k–o: cordate gametophyte. a–m, bar = 100 μm; n–o, bar = 1 mm.

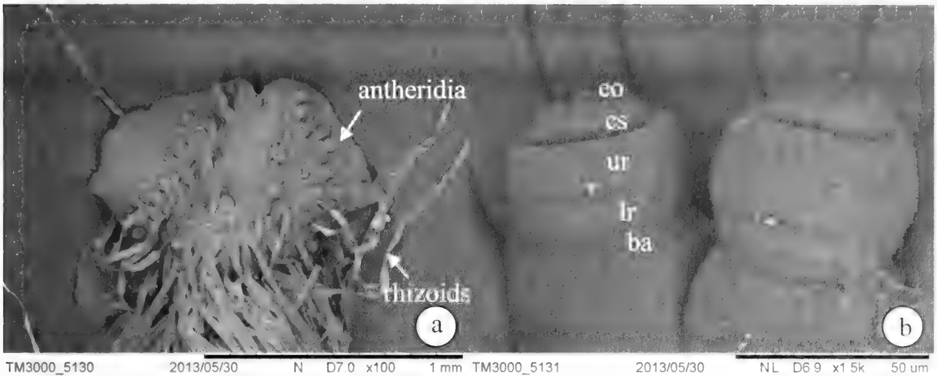

FIG. 3. Antheridia of *Aglaomorpha cornucopia*. a: on the ventral surface of gametophyte. b: cells of antheridium, ba=basal cell, ur= upper ring cell, lr=lower ring cell, cs= crescent-shaped cell, eo=elliptical opercular cell.

formed. When hermaphroditic gametophytes were old, they possessed only empty antheridia.

Female or hermaphroditic gametophytes did not produce sporophytes until 13 weeks. Sporophyte production reached a stable and maximum rate (64%) after 23 weeks. The mean time to sporophyte production was 17 weeks. Each young sporophyte consisted of a foot that separated the tissue from the gametophyte (Fig. 5).

The C-values (genome size) of gametophyte cells were half those of sporophyte cells (Fig. 6).

The first several sporophyte fronds were simple, cuneate at base, with single or forked free veinlets. Subsequent fronds had oblong blades, with pinnately free veinlets, followed by the production of leaves with areolate venation (Fig. 7).

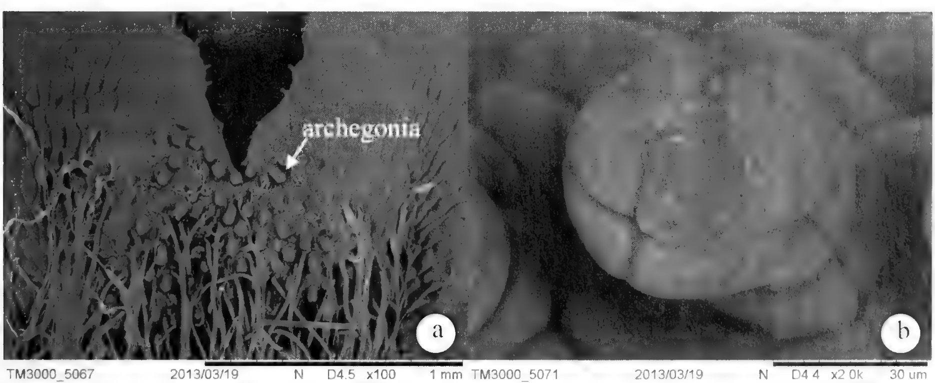

FIG. 4. Archegonia of *Aglaomorpha cornucopia* a: on the anterior cushion of gametophyte. b: archegonium.

Table 1. Sexual expression (%) and gametophyte size (mm) of *Aglaomorpha cornucopia* at different times in culture. Number of gametophytes bearing sporophytes in parentheses. A: asexual; M: male; F; female; H: hermaphroditic.

Size	6wk				7wk				9 wk				11 wk				13 wk			
(mm)	A	M	F	H	A	M	F	H	A	M	F	H	A	M	F	H	A	M	F	H
<0.5	41	-	-	-	40	-	-	-	18	10	-	-	3	8	-	-	-	13	-	-
0.5-1.0	49	-	-	-	31	2	-	-	14	11	3	-	2	12	-	-	-	13	-	-
1.1-2.0	10	-	-	-	17	5	1	-	10	5	7	-	1	15	4	2	-	17	-	1
2.1-3.0	-	-	-	-	-	-	4	-	1	-	12	-	1	3	16	3	-	9	2	4
3.1-4.0	-	-	-	-	-	-	-	-	-	-	8	-	1	-	6	2	-	-	5 (1)	5 (1)
4.1-5.0	-	-	-	-	-	-	-	-	-	-	1	-	-	-	8	1	-	-	7 (1)	-
>5.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	2	-	-	20 (8)	4 (1)
Sum	100	-	-	-	88	7	5	-	43	26	31	-	8	38	44	10	-	52	34 (10)	14 (2)

Discussion

Conservation of rare species is an important aspect in the global maintenance of biodiversity, yet, little experimental work has been completed on the prospects of spore banking in rare tropical ferns. The goal of this study was to explore the reproductive biology of the endangered *Aglaomorpha cornucopia* and to evaluate the efficacy of cold storage as a method for prolonging spore longevity.

Spore number and reproduction mode.—Reproductive systems vary in ferns. Whereas most taxa are sexual outcrossers, a large number of species reproduce asexually through apogamy or other means. Spore number per sporangium is generally considered a primary indicator of reproductive mode for most leptosporangiate ferns. Sexual taxa usually have 64-spored sporangia, and apogamous taxa have 32-spored sporangia (Huang *et al.*, 2006; Knobloch, 1967). This pattern also fits *Aglaomorpha cornucopia*, which had 64 spores per sporangium and reproduced sexually, as evidenced by its sporophyte formation (the clear separation between gametophyte and sporophyte) and the genome size difference observed between the sporophyte and gametophyte. Another congener, *A. meyeniana*, also has 64-spored sporangia and reproduces sexually (Ko *et al.*, 2004; Nayar, 1965).

Spore viability.—Although both fresh spores and spores that had been in cold storage for one-year achieved 100% germination, the one-year-old spores displayed delayed germination relative to fresh spores. These results indicate that spores of this species can be stored at cold temperature for at least up to one year. The dried spores of many ferns can remain dormant for several years, and longevity may be improved by reducing storage temperature. Spore banking holds the potential for long-term *ex situ* germplasm storage (reviewed by Pence, 2004, 2008). Additional work should investigate the viability of spores of *A. cornucopia* stored under additional treatments and for longer periods.

Spore germination and gametophyte development.—Nayar and Kaur (1971) defined several types of spore germination and gametophyte development.

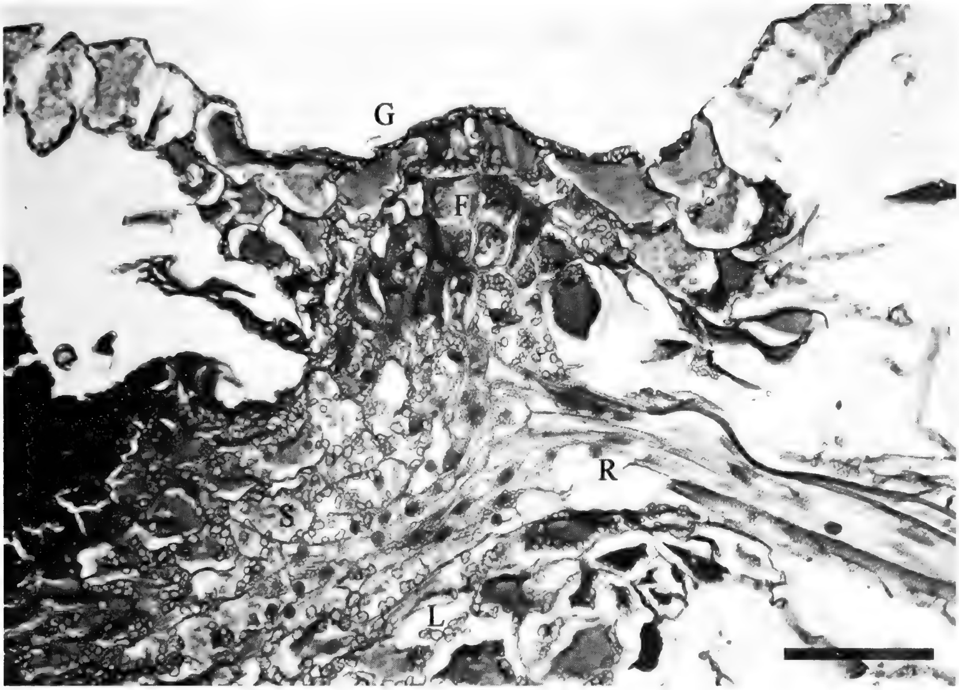

FIG. 5. Section of a gametophyte bearing young sporophyte (lower part) of *Aglaomorpha cornucopia*. A clear boundary between gametophytic tissue (G) and the foot (F) of young sporophyte were observed. (L) primary leaf; (R) primary root; (S) shoot. Bar = 100 μm.

Spore germination in *A. cornucopia* is of the *Vittaria*-type, and gametophyte development follows the *Drynaria*-type. These are the most common germination and development types in the Polypodiaceae. Other than the *Drynaria*-type of gametophyte development, other development types have been observed in some epiphytic members of the Polypodiaceae (Chiou and Farrar, 1997). We found that unicellular papillate hairs were scattered on gametophytes, and this is likely a stable characteristic of the Polypodiaceae (Nayar and Kaur, 1971).

The mature gametophyte of *A. cornucopia* is a typical cordate type. Once the cordate gametophyte sexually produced a young sporophyte, the gametophyte began necrosis. The life span of observed gametophytes of *A. cornucopia* was less than 6 months and could be classified as cordiform-annual following the definition by Farrar *et al.* (2008). The cordiform-annual gametophyte has been reported to be typical of terrestrial fern species (Farrar *et al.*, 2008); however, it has also been reported in many other epiphytic ferns of the Polypodiaceae (Chiou and Farrar, 1997; Ganguly *et al.*, 2009; Pérez-García *et al.*, 1998; Reyes *et al.*, 2003) as in *A. cornucopia*.

Gametangia.—Nayar and Kaur (1971) reported that the leptosporangiate type of antheridium commonly produces only 16 to 32 spermatozoids.

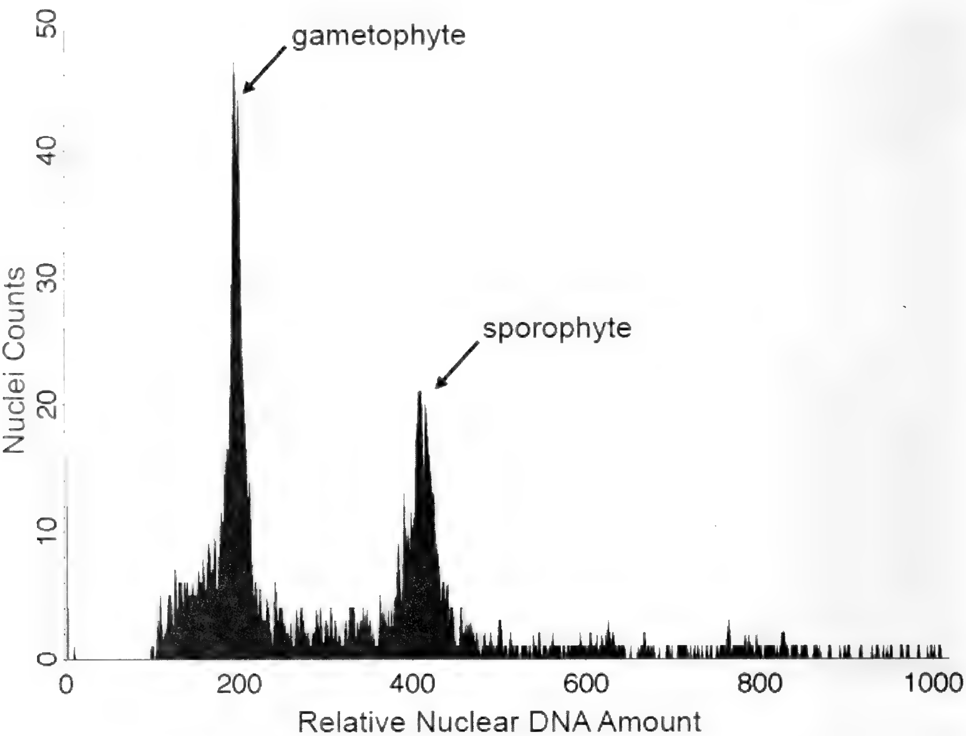

FIG. 6. Relative C-value (genome size) of gametophyte and sporophyte in *Aglaomorpha cornucopia*.

Gametophytes of *A. cornucopia* produced 32 spermatozoids/antheridium in this study. It is worth noting that sexual haploid and apogamous triploid gametophytes of *Pteris fauriei* Hieron. produced 64- and 32-spermatozoid antheridia, respectively (Huang *et al.*, 2006). In addition, the gametophytes of three apogamous *Pteris* (*P. cretica* L., *P. pellucidifolia* Hayata, and *P. wulaiensis* C. M. Kuo) were also observed to produce 32-spermatozoid antheridia (Huang *et al.*, 2011). However, no such data have been reported in other species of Polypodiaceae. More work is needed to determine if the spermatozoid number/antheridium in ferns is related to phylogeny and/or indicative of reproductive mode.

Apart from the central spermatogenous cell which eventually develops into spermatozoids, an antheridium possesses a set of jacket cells that envelope this central spermatogenous zone (Leung and Näf, 1979). The number of antheridial jacket cells varies among leptosporangiate ferns. Antheridia with jackets of ≥5 cells have been described in most early leptosporangiate ferns, such as Osmundaceae, Hymenophyllaceae, and some tree ferns (Chen *et al.*, 2008; Huang *et al.*, 2003a; Momose, 1967; Nayar and Kaur, 1971). In contrast to early leptosporangiate ferns, the typical antheridium of polypods has a jacket of three cells, a basal cell, a ring cell, and a cap cell (Momose, 1967; Nayar and

FIG. 7. Frond profiles of young sporophyte of *Aglaomorpha cornucopia*. a–d: first frond with free single to forked veinlet. e–k: subsequent fronds with free pinnate veinlets, l–o: subsequent fronds with areolae. a–b, bar = 2 mm; c–k, bar = 5 mm, l–o, bar = 1 cm.

Kaur, 1971; Pérez-García and Mendoza-Ruiz, 2004, 2005, 2006; Raghavan, 1989; Zhang *et al.*, 2011). In *A. cornucopia* we found that jackets of the antheridia were composed of 5 cells: an opercular cell and a crescent-shaped cell that made up the cap cell, and the ring cell divided into upper ring cell, lower ring cells, and basal cell. This is unlike the typical 3-celled-jacket per antheridium previously reported in other species in the Polypodiaceae by Nayar and Kaur (1971) and Leung and Näf (1979). Indeed, we believe this to be the first report of such antheridia within polypods.

The 4-celled neck of the archegonium of *A. cornucopia* is similar to other species of Polypodiaceae. According to Momose (1967), the cell numbers of each tier of the archegonium neck in polypod ferns are 3–7, whereas they are 4–9 in other leptosporangiate ferns.

Sexual expression and antheridiogen.—Homosporous ferns have the potential to produce hermaphroditic gametophytes. Some mechanisms have been documented to avoid the occurrence of intragametophytic selfing, such as high genetic load, and/or temporal separation of male and female gametangia on a single gametophyte (Chiou *et al.*, 2002, 2003; Klekowski, 1982; Peck *et al.*,

1990). Gametophytes of *A. cornucopia* initially are unisexual (male or female), and then some of them became hermaphroditic with further growth. Although, the hermaphroditic gametophytes simultaneously possessed both antheridia and archegonia, bisexual gametophytes possessed antheridia that had already released their spermatozoids. As such, these gametophytes are functionally female. In addition, more than 85% of the gametophytes were unisexual under our culture conditions. Therefore, intergametophytic mating is more likely for *A. cornucopia*, as evidenced by its sexual expression.

Antheridiogens, a kind of pheromone, can be secreted by large, mature gametophytes in some ferns to promote antheridium formation of other later-germinated gametophytes (reviewed by Schneller, 2008). This pheromone has been found in some species of Polypodiaceae (Chiou *et al.*, 1997). In the current study, most male gametophytes (ca. 90%, width $<$ 2.0 mm) were filamentous or spathulate and smaller than female and hermaphroditic gametophytes, which were heart shaped. This phenomenon may indicate the occurrence of an antheridiogen system in this species.

Gametophyte cultures are easily contaminated by other fern spores, which may germinate in the same container. The characteristics of young sporophytes, demonstrated in this report, provide a practical post-hoc examination of the target species and confirm the accuracy of the experiment. In addition, knowledge of early frond development can also serve the purpose of helping to identify wild populations of the species that may not have fully developed fronds. This information may facilitate demographic and ecological studies of this rare fern in the wild that are crucial for developing conservative strategies *in situ* (Testo and Watkins, 2013).

Conclusions.—In this study, *Aglaomorpha cornucopia*, a rare endemic fern from the Philippines, was cultured from spores. Spore germination, gametophyte development, and the formation of young sporophytes were closely observed and illustrated in detail. Results show that the sporophytes reproduced sexually, as evidenced by spore number/sporangium, the genome sizes of sporophytes were double those of gametophytes, and there was a clear separation between the tissues of the gametophyte and sporophyte. Intergametophytic mating is more likely than intragametophytic selfing as inferred by sexual expression and indirect evidence of an antheridiogen system in this species. Spores remained viable after one-year of storage at 3°C. These results suggest that spores of *A. cornucopia* could be stored under cold storage for at least one year for ultimate use in *ex situ* conservation programs.

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Independent Gametophytes of *Hymenophyllum wrightii* in North America: Not as Rare as We Thought

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Abstract.—*Hymenophyllum wrightii* is a filmy fern known primarily from Japan and Korea. In North America, it is known as a sporophyte only in Haida Gwaii (Queen Charlotte Islands), British Columbia, Canada. Rare independent, filmy fern gametophytes found from the late 1950s and onward at a handful of locations in British Columbia and southeastern Alaska were presumed to be *H. wrightii*. Our 2006 surveys in southeastern Alaska determined that these gametophytes are common within specific habitats, and our survey in 2008 found gametophyte populations on the Olympic Peninsula in Washington State—the first report of *H. wrightii* in the contiguous United States. Samples from gametophyte populations from southeastern Alaska, British Columbia and Washington show no variation in *rbcL* or *rps4-trnS* sequence and are similar to sequences from Asian *H. wrightii* sporophytes, providing corroborating evidence of the identity of these independent gametophyte populations.

Key Words.—asexual, *Hymenophyllum wrightii*, independent gametophyte, Olympic Peninsula, southeastern Alaska, Vancouver Island

Hymenophyllum wrightii Bosch is a filmy fern (Hymenophyllaceae) found primarily in Japan and Korea, but with small disjunct populations on the northwest coast of North America. In North America, *H. wrightii* sporophytes are known from a few sites in Haida Gwaii (Queen Charlotte Islands), British Columbia, Canada (Iwatsuki, 1961; Persson, 1958; Taylor, 1967). Isolated, apparently asexual gametophyte populations have been reported over a wider area, including one site in southeastern Alaska (Taylor, 1967), six sites in Haida Gwaii and the area near Prince Rupert, British Columbia (Schofield, 1962; Taylor, 1967), and three sites on Vancouver Island (Cordes and Krajina, 1968). Since those initial reports only a few additional gametophytes have been collected (University of British Columbia Herbarium; Figs. 1–3). Because of its apparent rarity, *H. wrightii* was designated as a Sensitive Species by the USDA Forest Service, Alaska Region, during the 1990s (Goldstein *et al.*, 2009).

Identifying ferns to genus and species based on gametophyte morphology is difficult (de Groot *et al.*, 2011; Li *et al.*, 2009; Schneider and Schuettpelz, 2006; Watkins *et al.*, 2007) but identification to genus and subgenus is often possible

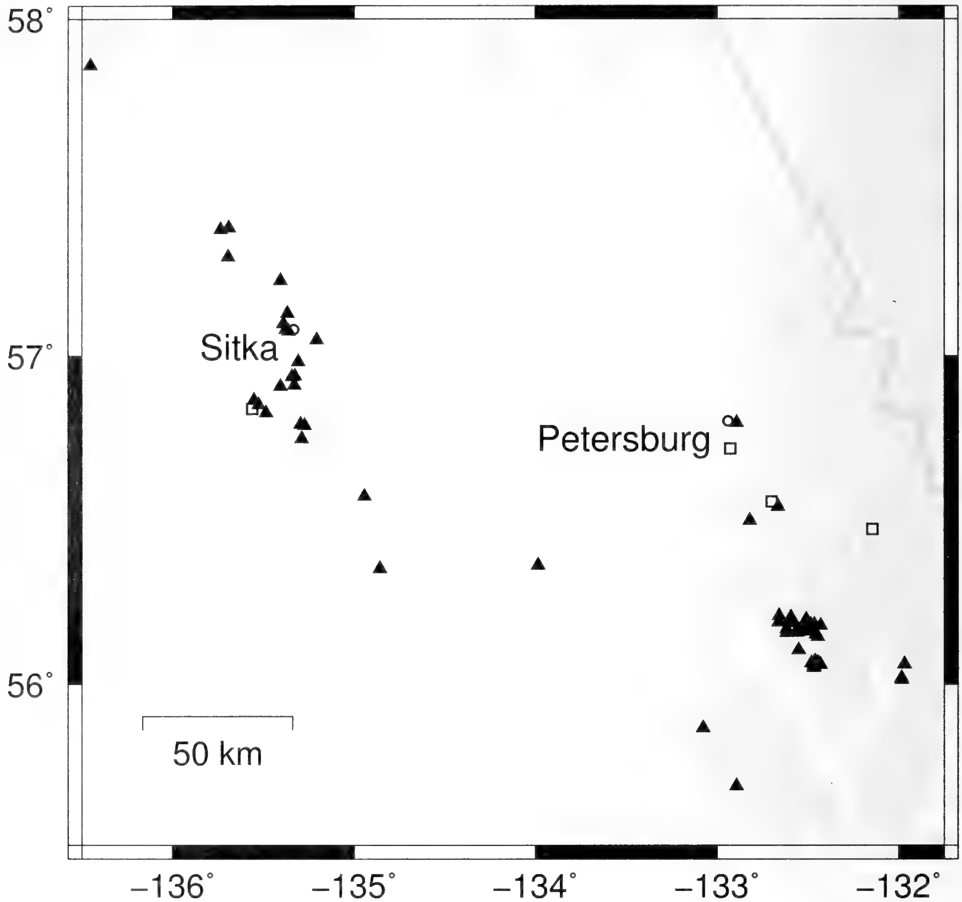

FIG. 1. Map of southeastern Alaska showing locations where *Hymenophyllum wrightii* gametophytes were found during and since 2006 (black triangles) and locations where gametophytes were reported or collected prior to 1980 (open squares).

in taxonomic groups possessing distinctive gametophyte morphologies (Farrar *et al.*, 2008). These populations of gametophytes are assumed to be *H. wrightii* because it is the only filmy fern known from western North America and because the North American gametophytes do not differ morphologically from gametophytes found growing among *H. wrightii* sporophytes from Japan and Haida Gwaii (Taylor, 1967). Furthermore, their gemmae are morphologically diagnostic of subgenus *Mecodium* to which *H. wrightii* belongs (Raine *et al.*, 1991). The gametophytes of these populations are slow-growing, long-lived, branching, ribbon-like thalli, and they produce gemmae at their margins (Fig. 4). They form dense tangled mats, but are small, grow in dark sheltered locations, and are easily overlooked.

Hymenophyllum wrightii grows on a variety of substrates in different parts of its range. Sporophytes in Japan are found on the bases of large trees in humid

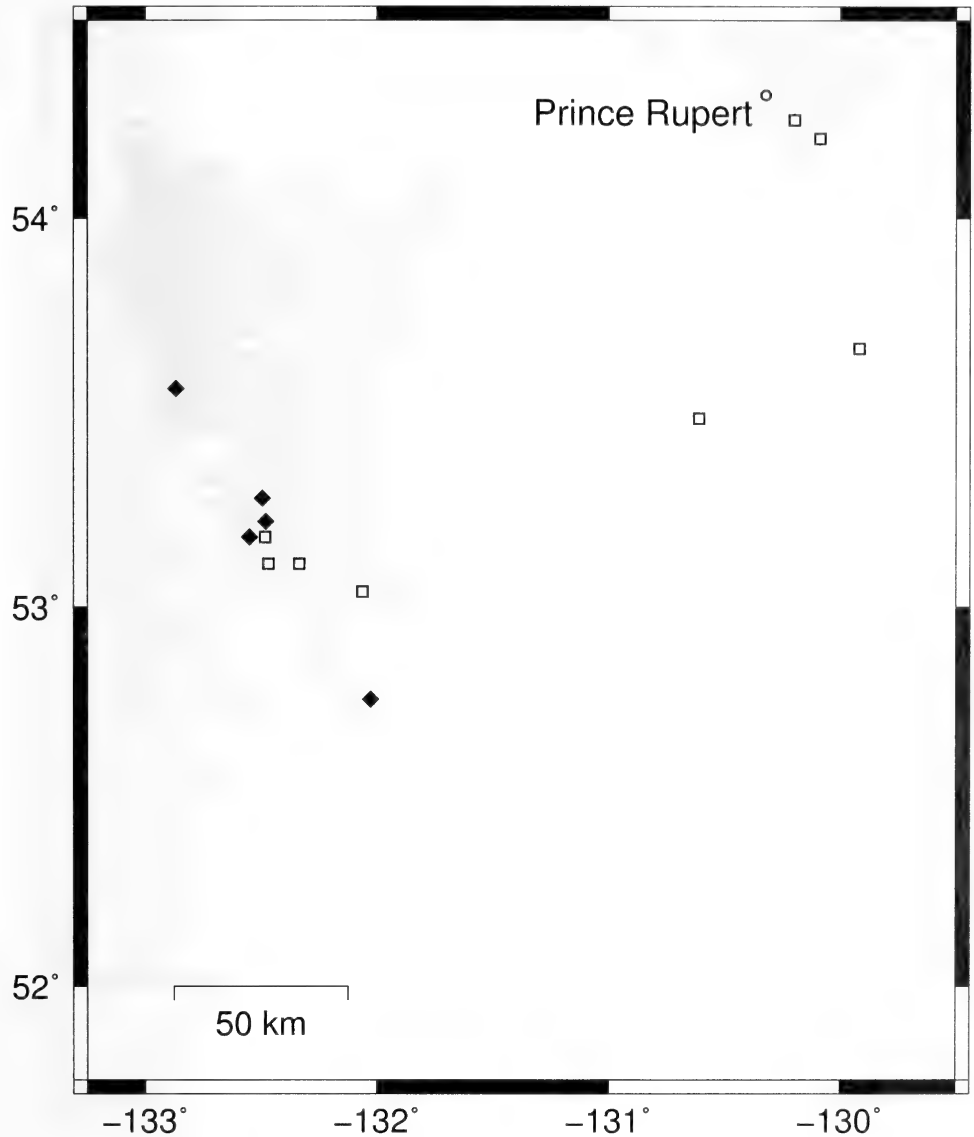

FIG. 2. Map of Haida Gwaii and the area surrounding Prince Rupert, British Columbia showing locations where *Hymenophyllum wrightii* sporophytes have been reported or collected (black diamonds) and *Hymenophyllum wrightii* gametophytes were reported or collected prior to 1980 (open squares).

forests and on moist shaded rocks and cliffs at elevations up to 1000 meters. The sporophyte-producing populations in Haida Gwaii are found on dark, shady, wet cliffs near sea level. The gametophytes collected in the 1960s through 1980s in Haida Gwaii, near Prince Rupert, and in southeastern Alaska were also growing on deeply shaded, wet rock outcroppings beneath

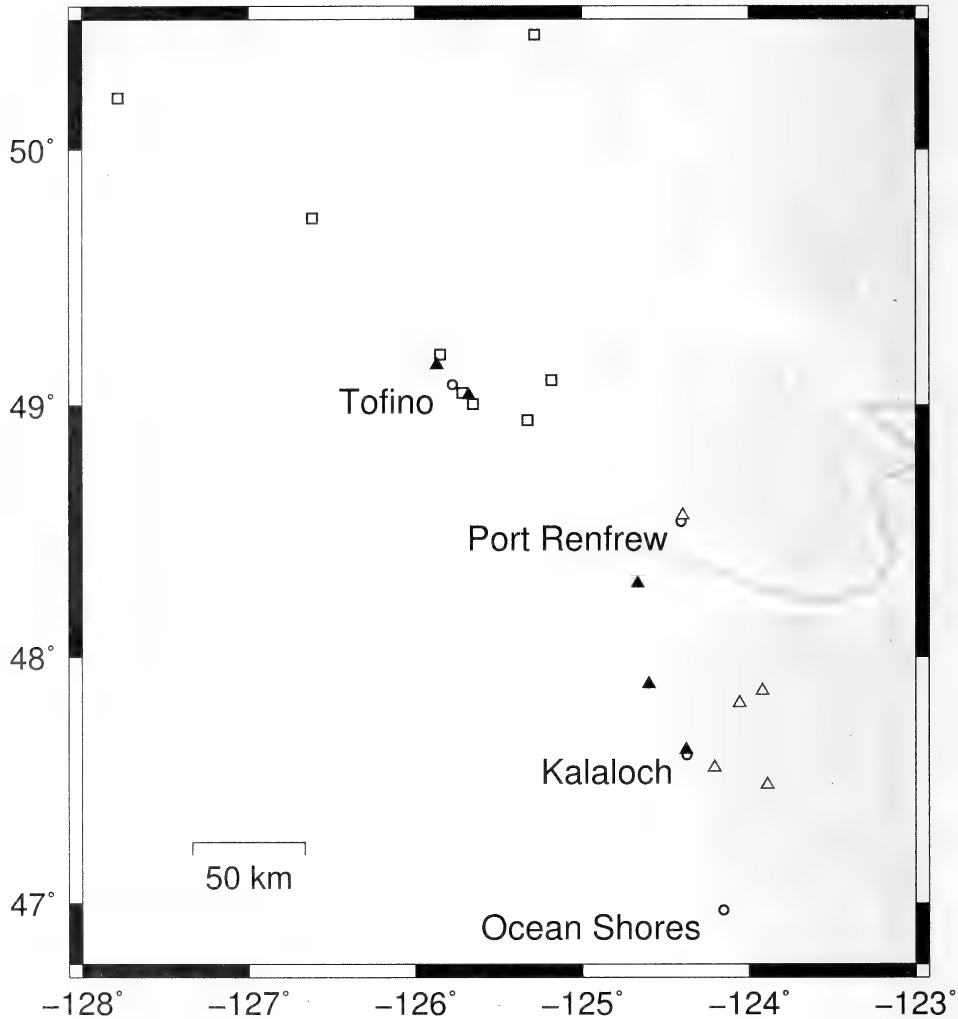

FIG. 3. Map of southeastern Vancouver Island and the Olympic Peninsula showing locations where *Hymenophyllum wrightii* gametophytes were found in 2008 (black triangles), areas that were searched unsuccessfully in 2008 (open triangles), and locations where gametophytes were reported or collected prior to 1980 (open squares).

overhangs or in wet cliff crevices near the coast or in stream canyons, although one collection was found on an overturned stump and another grew on the underside of a fallen Douglas fir, spanning a stream (Taylor, 1967; University of British Columbia Herbarium). On Vancouver Island, gametophytes were found on rotting wood or on old bark of Sitka spruce within 500 m of the coast (Cordes and Krajina, 1968).

Because the potential habitats of *H. wrightii* gametophytes are common throughout the Pacific Northwest, and because the gametophytes are so easily overlooked, it seems likely that many unidentified populations could

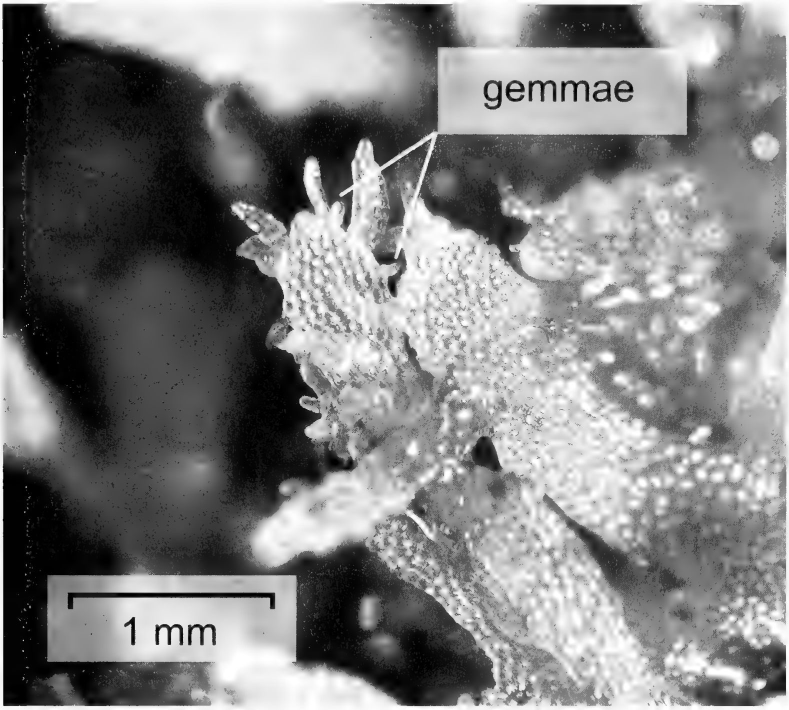

FIG. 4. *Hymenophyllum wrightii* gametophytes showing branching morphology and gemmae growing at the thallus margins.

exist. However, because logging may have impacted the dark shaded habitats where these plants were first reported 40 years ago, it is possible that some old sites have been lost. To get better data on the current status of this species, we set out to search specifically for *H. wrightii* populations in southeastern Alaska, on Vancouver Island, British Columbia, and on the Olympic Peninsula in Washington State. Our goals were to determine: 1) whether gametophyte populations are indeed rare or merely overlooked; 2) whether previously reported populations could be relocated; 3) whether additional populations could be found in apparently suitable habitats beyond the known range, and; 4) whether the North American gametophytes are indeed *H. wrightii.*

MATERIALS AND METHODS

Surveys.—In July of 2006, we intensively surveyed areas in southeastern Alaska near Sitka and Petersburg where gametophytes had previously been reported, as well as in other areas having potential habitats. During July of 2008, we surveyed sites on the west coast of Vancouver Island, British Columbia, including the Tofino area, where gametophytes were previously reported as well as further south on Vancouver Island near Port Renfrew. We

also surveyed the west coast of the Olympic Peninsula in Washington State as far south as Ocean Shores, Grays Harbor County.

Our efforts focused on microhabitats similar to those where gametophytes were previously reported: on wet tree bases, beneath overturned stumps, and on rock outcrops in deeply shaded rainforest. Light levels were so low as to require the use of flashlights and hand-lenses to distinguish *H. wrightii* gametophytes from the surrounding mosses and liverworts. Most areas searched were at low elevations within 1.5 km of the coast. However, on the Olympic Peninsula, we also searched sites up to 30 km inland along the Hoh, Queets, and Quinault Rivers.

DNA extraction, amplification, and sequencing.—A single living gametophyte was separated from each sample, manually cleaned of bryophytes, visible algae, or other contaminants, and washed in distilled water. Each gametophyte was ground with a pestle in a 1.5 µL tube on ice, and genomic DNA was extracted using a CTAB method (Doyle and Doyle, 1987).

We PCR amplified approximately 1300 bp of the *rbcL* gene using primers F1F and F1379R (Little and Barrington, 2003) and approximately 1000 bp of the *rps4* gene and *rps4* to *trnS*-GGA spacer (Small *et al.*, 2005). PCR amplification followed Der *et al.* (2009) for *rps4-trnS*, and Wolf *et al.* (1994) for *rbcL*. Fragments were sequenced in both directions using the PCR primers as well as filmy fern specific internal primers H1R1 and H1F1 for *rbcL* (Ebihara *et al.*, 2003), in an ABI 377 sequencer using the DYEnamic ET Terminator (Amersham Biosciences). Sequences for each individual were joined in Sequencher 4.5 (Gene Codes Corporation) and compared to other *rbcL* and *rps4-trnS* sequences using online BLAST (Altschul *et al.*, 1990).

Other barcoding markers or combinations of markers, such as *rbcL* and *trnL-trnF*, have been proposed as more effective at identifying ferns to species (Chen *et al.*, 2013; de Groot *et al.*, 2011; Li *et al.*, 2009). However, *rbcL* and *rps4-trnS* sequences for many *Hymenophyllum* species were available in GenBank as a result of phylogenetic studies of filmy ferns (Ebihara *et al.*, 2003; Hennequin *et al.*, 2006, 2010), so we selected these markers in order to maximize the number of species comparisons.

Results

Surveys.—We found gametophytes at the locations near Sitka and Petersburg, Alaska, where they had previously been reported as well as at approximately 60 other locations in southeastern Alaska (Fig. 1). Gametophytes were found at two locations near Tofino on Vancouver Island, including Meares Island (Fig. 3), one of the sites where gametophytes had previously been reported (Cordes and Krajina, 1968). On the Olympic Peninsula, we found gametophytes at three sites extending as far south as Kalaloch (Fig. 3). No *H. wrightii* sporophytes were found at any of the survey sites, nor have any sporophytes been produced in any of our live cultures in the years since these collections were made.

TABLE 1. *Hymenophyllum wrightii* gametophyte populations included in DNA sequencing. Because no sequence variation was found among populations only a single *rbcL* and *rps4-trnS* sequence representing each region (southeastern Alaska, Vancouver Island, and the Olympic Peninsula) was deposited in GenBank.

Collection	Location	Voucher	Accession
Duffy 08004	Rain Forest Trail near Tofino, Vancouver Island, British Columbia	UTC255727	
Duffy 08005	Rain Forest Trail near Tofino, Vancouver Island, British Columbia	UTC255728	
Duffy 08008	Rain Forest Trail near Tofino, Vancouver Island, British Columbia	UTC255729	
Duffy 08009	Rain Forest Trail near Tofino, Vancouver Island, British Columbia	UTC255730	
Duffy 08011	Big Trees Trail, Meares Island, British Columbia	UTC255731	JN585965 (*rbcL*), JN585968 (*rps4-trnS*)
Duffy 08013	Shi-Shi Beach Trail near Neah Bay, Washington	UTC255732	JN585966 (*rbcL*), JN585969 (*rps4-trnS*)
Duffy 08014	Shi-Shi Beach Trail near Neah Bay, Washington	UTC255733	
Duffy 08015	Third Beach near La Push, Washington	UTC255734	
Stensvold and Farrar 8319	Mitkof Island (South) near Banana Point, Alaska	UTC256069	
Stensvold and Farrar 8320	Mitkof Island (North) near Petersburg, Alaska	NA	
Stensvold and Farrar 8329	Biorka Island near Symonds Bay, Alaska	UTC256068	
Stensvold and Farrar 8334	Krestof Island near Olga Point, Alaska	UTC256071	
Stensvold and Farrar 8335	Chicagof Island near Bradshaw Cove, Alaska	UTC256070	
Stensvold and Farrar 8338	Kruzof Island near Sukoi Inlet, Alaska	UTC255737	JN585964 (*rbcL*), JN585967 (*rps4-trnS*)

DNA sequencing.—We sequenced full or partial *rbcL* and *rps4-trnS* from six samples from southeastern Alaska, five from Vancouver Island, and three from the Olympic Peninsula (Table 1). These sequences, representing populations from the known northern and southern range extents of *H. wrightii* in North America show no variation at either *rbcL* or *rps4-trnS*.

The *rbcL* sequences of these North American gametophytes differ from an Asian *H. wrightii* sporophyte sample (GenBank: AB083277.1; Ebihara *et al.*, 2003) at only two nucleotide positions. The North American *rps4-trnS* sequences differ from the Asian sample (GenBank: AY775430.1; Hennequin *et al.*, 2006) at only three nucleotide positions. One of those three nucleotides is ambiguous and could not be called with confidence in any of the North American sequences (despite otherwise clean sequence reads). The other two differences are a 'TA' missing from a dinucleotide repeat (six repeats in the North American sequences and seven in the Asian sequence) in a region that appears to be highly variable among *Hymenophyllum* species.

The North American *H. wrightii* gametophytes show more sequence similarity to *H. wrightii* than to sequences in GenBank from other *Hymenophyllum* species. The next closest *rbcL* match is an accession of *H. polyanthos* from Japan (GenBank: AB083276.1; Ebihara *et al.*, 2003) with 12 nucleotide differences and the next closest *rps4-trnS* match is *H. inaequale* from La Réunion (GenBank: AY095122.1; Hennequin *et al.*, 2003) with approximately 30 nucleotide differences.

Discussion

Our survey findings suggest that *H. wrightii* is more common and widespread on the northwest coast of North America than previously thought. We identified many additional gametophyte populations within the previously known range and found populations outside that range. *Hymenophyllum wrightii* gametophytes were located at previously reported sites near Sitka and Petersburg and on Vancouver Island, and found at locations in the surrounding areas. Significantly, populations of *H. wrightii* gametophytes were found on the Olympic Peninsula in Washington State, extending the known range of this species southward by approximately 200 km and providing the first reports of *H. wrightii* in the contiguous United States.

The surveys on Vancouver Island and the Olympic Peninsula were not as intensive or exhaustive as those in southeastern Alaska, so the results in these areas cannot be directly compared and our survey methods were not systematic—the dense clustering in certain areas (Fig. 1) is the result of focused collecting efforts. However, it appears that *H. wrightii* gametophytes are not as common on Vancouver Island or the Olympic Peninsula as they are in Alaska, but the ease with which we found new populations suggests that additional previously unknown populations may be found in appropriate microhabitats.

As a result of these surveys we now have better information on the habitats and microhabitats where *H. wrightii* is most likely to be found. This species grows on wet substrates in dark hollows of rotten fallen trees or at the bases of dead standing trees, and beneath the exposed roots and on the bases of mature conifers. It prefers the darkest areas, where competition with bryophytes is minimal. These habitats occur under the closed canopy of shady rainforests within a few hundred meters of the coast.

We did not search all habitats and sites exhaustively, so we cannot be certain that we did not overlook some *H. wrightii* occurrences, but certain commonalities were observed among the habitats where it was found. Gametophytes were not found at sites over 1.5 km inland, even though these inland sites were chosen specifically because they receive similar amounts of rainfall to southeastern Alaskan sites. Despite the high amount of rainfall in the Hoh, Queets, and Quinault rainforests, the dark inner surfaces of likely looking stumps were dry compared to stumps in coastal forests receiving less rainfall, suggesting that perhaps coastal humidity or temperature moderation play a role in creating suitable habitat for *H. wrightii* gametophytes. Other

environmental factors such as soil type or substrate pH may be important and warrant further investigation. We also did not find gametophytes in sites that had been recently logged or burned. In several areas, we inspected the rotting remains of burned trees but did not find gametophytes. As we moved south on the Olympic Peninsula, patches of wet, dark forest became more difficult to find, but we found gametophytes as far south as Kalaloch, Jefferson County.

Additional populations may exist at microsites further south along the Pacific coast, along parts of Puget Sound, and further west along the Alaskan coast. However, it appears that these gametophytes require high levels of moisture and dark, temperature-moderated environments. Based on the habitats where they have and have not been found, as well as observations of plants maintained in culture, the gametophytes may not tolerate repeated drying and seem to compete poorly with mosses and liverworts in brighter lighting. Other long-lived filmy fern gametophytes are frequently found in both desiccated and frozen states (Farrar, 1998), so a single event may not prevent *H. wrightii* gametophytes from inhabiting a site, but the average conditions in a particular location may not allow sufficient assimilation through photosynthesis to exceed loss due to respiration in the long-term. Suitable habitat likely becomes scarce in Puget Sound or further south along the Pacific coast in Washington due to low moisture levels, and in Alaska west of Kodiak due to a lack of rainforest.

Through DNA sequencing, we confirmed that the *Hymenophyllum wrightii* gametophytes growing on the west coast of North America are genetically similar to Asian *H. wrightii* sporophytes at two chloroplast loci. The sequences from North American and Asian plants are not identical, but they are much more similar to each other than either is to other available *Hymenophyllum* species sequences. This genetic evidence corroborates the previous identification of these North American gametophytes as *H. wrightii* based on their morphology—though it cannot rule out the possibility of hybrid origins or the possibility that they are gametophytes of some *Hymenophyllum* species that is not represented in GenBank. Further study, including sequencing sporophytes from Haida Gwaii is still necessary.

Hymenophyllum wrightii is the only fern reported to form long-lived, gametophyte-only populations in western North America, but other ferns share this unusual lifecycle, including *Crepidomanes intricatum* (Farrar) Ebihara & Weakley (Hymenophyllaceae) and *Vittaria appalachiana* Farrar & Mickel (Vittariaceae) in the eastern United States. However, for those species no sporophytes have been found and it seems likely that these species are no longer (or were never) able to produce them (Farrar, 1990, 1992, 1998; Farrar and Mickel, 1991). *Hymenophyllum tayloriae* Farrar & Raine (Hymenophyllaceae) in the southern Appalachian Mountains and Appalachian plateau produces independent gametophyte colonies, some of which also produce juvenile sporophytes, but mature sporophytes have not been found (Raine *et al.*, 1991). *Callistopteris baueriana* (Endl) Copeland (Hymenophyllaceae) in Hawai'i, is found as dense mats of gametophytes which only produce sporophytes in wetter, higher altitude locations (Dassler and Farrar, 1997).

In the case of *H. wrightii*, it is not clear whether the apparent absence of sporophytes outside of Haida Gwaii is due to some habitat limitation, or whether these gametophytes are genetically incapable of producing sporophytes (as suggested by the lack of sporophytes in culture). This raises questions of how the North American gametophyte populations were established. Are they the result of recent spore dispersal from the sporophyte populations in Haida Gwaii or elsewhere, or are they reproducing only asexually and dispersing via gemmae? If they are currently limited to asexual reproduction, how and when were the populations established? Further work, including DNA sequencing of sporophyte samples from Haida Gwaii and using genetic markers that are variable among populations, will be required to distinguish among these possibilities.

A better understanding of the relationship between the Haida Gwaii sporophytes and the widespread gametophyte populations will be important in management and conservation of this species. Based on our surveys in southeastern Alaska, *H. wrightii* was removed from the Forest Service's Alaska Region Sensitive Species List in 2009 (Goldstein *et al.*, 2009) on account of its great abundance, wide distribution, and stable populations in the Tongass National Forest. In British Columbia, *H. wrightii* is identified as a 'yellow' listed species rated as 'Vulnerable' to 'Apparently Secure' (B. C. Conservation Data Centre). Sporophytes and gametophytes are managed as a single entity, though they differ greatly in their distribution. *Hymenophyllum wrightii* gametophytes on the Olympic Peninsula do not appear to be nearly as common or widespread as in southeastern Alaska, but further study is warranted to determine appropriate protections.

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Shorter Notes

Huperzia miyoshiana **(Makino) Ching new to Oregon, U.S.A.**—Saddle Mountain and several nearby peaks in the northern Coast Range of Oregon are well known for endemic and disjunct elements in their vascular and bryophyte floras (Chambers, Madroño 22:105–160. 1973; Chambers, Madroño 22:278–279. 1974; Detling, N.W. Sci. 28:52–60. 1954; Schofield and Godfrey, Hattori Bot. Lab. 46:285–288. 1979). These rugged basalt peaks, with elevations of 850–990 m and located 5–20 km from the Pacific Ocean, protrude abruptly above the surrounding landscape and intercept large amounts of precipitation and summer fog. The Pacific fir-moss, *Huperzia miyoshiana*, recently confirmed from these peaks, is a new record for the state of Oregon, and is the farthest south known locality for the species in North America.

Wilfred Schofield, former professor of bryology and plant geography at the University of British Columbia (UBC; Harpel, Bryologist 112:257–267. 2009) collected a specimen that he identified as *Lycopodium selago* on Saddle Mountain in 1978. The first author collected a similar plant in 2007 on Sugarloaf Mountain, ca. 18 km SW of Saddle Mountain. The Schofield specimen remained unknown to Oregon botanists until 2011, when UBC's digital herbarium records were posted on the Consortium of Pacific Northwest Herbaria website at the University of Washington (http://www.pnwherbaria.org). The specimen at UBC was annotated in 2007 by Christopher Sears as *Huperzia haleakalae* (Brackenridge) Holub, and reannotated in 2012 by Curtis Bjork and Jamie Fenneman as *H. chinensis* (Herter ex Nessel) Ching. Recent examination of both specimens by the authors, and comparison with material from NY annotated by J. M. Beitel, co-author of treatments of *Huperzia* for the Pacific Northwest and North America (Beitel and Ceska, pp. 118 *in* Douglas et al., Vasc. Pl. British Columbia 3. 1991; Wagner and Beitel, Lycopodiaceae. Fl. N. Amer. 2:18–37. 1993), indicate that characters from both are consistent with fertile *Huperzia miyoshiana*. This confirms that *H. miyoshiana* is new to the flora of Oregon, extending its known latitudinal range in North America south by ca. 115 km. The closest known collections are from Olympic National Park and Mount Rainier National Park (Consortium of Pacific Northwest Herbaria database).

Four species of *Huperzia* are known from western North America—*H. haleakalae, H. miyoshiana, H. occidentalis* (Clute) Kartesz & Gandhi, and *H. selago* (Linnaeus) Bernhardi ex Schrank & Martius (Wagner and Beitel, 1993). *Huperzia miyoshiana* closely resembles *H. haleakalae* but can be distinguished by several characters. *Huperzia miyoshiana* produces vegetative gemmae in 2 to 3 pseudowhorls at the end of annual growth, in contrast to *H. haleakalae* that has gemmae distributed throughout the mature portion of the shoot. The leaves of *H. miyoshiana* are somewhat longer and more spreading than those of *H. haleakalae*. Juvenile shoots of *H. miyoshiana* are sharply downcurved, in contrast to the erect or ascending juvenile shoots of *H.*

haleakalae, as well as those of *H. occidentalis* and *H. selago*. However, neither of the specimens of *H. miyoshiana* from Oregon includes juvenile shoots. Sterile interspecific hybrids between *H. miyoshiana* and *H. haleakalae* have been found elsewhere in the Pacific Northwest (Beitel, Amer. J. Bot. 73: 733–734 [Abstr.], 1986), but the spores of both Oregon collections, though somewhat immature, appear to be normal. In the Pacific Northwest, *H. miyoshiana* is generally a montane species, in contrast to the typically subalpine habitats of *H. haleakalae*. The only other species of *Huperzia* currently known from Oregon, *H. occidentalis*, has a scattered distribution across the northern edge of the state. In contrast to *H. miyoshiana*, it has only a single pseudowhorl of gemmae for each year's annual growth, and its lower stem leaves are longer, widespreading, and broadly oblanceolate.

In Oregon, typical habitat of *Huperzia occidentalis* is shaded, moist, conifer forest at low to mid elevations. In contrast, habitat for *H. miyoshiana* at the two known sites is cool, damp, north- and east-facing basalt outcrops within 20 km of the Pacific Ocean, exposed to strong winds, on very thin soil over bedrock. Associated species include *Abies amabilis, Tsuga heterophylla, Acer circinatum, Cladothamnus pyroliflorus, Castilleja chambersii,* and *Cardamine pattersonii.* These habitats are of great conservation interest because of the presence of many endemic or disjunct vascular plants and bryophytes. Because of the paucity of known sites in Oregon, *Huperzia miyoshiana* has been added to the Oregon Biodiversity Information Center's List 3, (http://orbic.pdx.edu/rte-species.html) for species in need of more information, but potentially threatened or endangered in Oregon.

Huperzia miyoshiana exhibits what has been variously called a North Pacific, amphi-Beringian, or Pacific North American-Pacific Asian distribution (Schofield, J. Hattori Bot. Lab 55:35–43. 1984), with a disjunct occurrence in Newfoundland (Brunton et al., Amer. Fern J. 82:63–66. 1992; Zhang and Iwatsuki, pp. 13–34 *in* Wu et al., Flora of China 2–3 (Pteridophytes). 2013). Saddle Mountain and other nearby peaks serve as refugia for a number of rare bryophytes that share the same distribution as *H. miyoshiana*, and that were considered by Schofield (Canad. J. Bot. 66:2673–2686. 1988) to be relictual fragments of a once more widespread periglacial and immediate post-glacial flora.

The annotation history of the Saddle Mountain specimen at UBC highlights recent nomenclatural confusion in North America between *H. miyoshiana* and *H. chinensis*. Some databases and floras for North America (e.g., NatureServe Explorer, http://www.natureserve.org/explorer; E-Flora BC, http://www.geog.ubc.ca/biodiversity/eflora/index.shtml; Douglas et al., Ill. Fl. British Columbia 5:276. 2000; and until recently corrected, USDA PLANTS Database, http://plants.usda.gov) treat *H. miyoshiana* as a synonym of *H. chinensis*. However, critical treatments of temperate *Huperzia* (Wagner and Beitel, 1993; Zhang and Kung, Acta Phytotax. Sin. 36:521–529. 1998; Zhang and Iwatsuki, pp. 13–34 *in* Wu et al., Fl. China 2–3 (Pteridophytes). 2013) maintain *H. miyoshiana* and *H. chinensis* as distinct species, based on differences in morphology and geography, and restrict the geographical range of *H. chinensis* to China. We

follow these recent interpretations because they include representatives of this species group from the Pacific Northwest.

Specimens Examined.—USA, Oregon, Clatsop County: (1) Saddle Mountain State Park, 19 km ESE of Seaside, 45°57′59.96N, 123°40′59.28W. Elev. ca. 865 m. Wilfred B. Schofield *s.n.*, 22 March 1978 (UBC); (2) Sugarloaf Mountain, 9 km ESE of Cannon Beach, 45°51′34.37N, 123°51′24.90W. Elev. ca. 850 m. John A. Christy *9957*, 21 May 2007 (OSC).

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